

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HADLACZKY et al.

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Examiner: Martin, J.

For: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR  
PREPARING ARTIFICIAL CHROMOSOMES

## DECLARATION PURSUANT TO 37 C.F.R. §1.132

The Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Carl Perez declare as follows:

1) I am currently Director of Projects at Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9. I have held this position since March 10, 1997. I earned a doctoral degree in biophysics at the University of California at Berkeley in August 1984.

2) In my position at Chromos Molecular Systems, Inc., (hereinafter Chromos) I have been extensively involved in projects designed to generate transgenic animals using satellite artificial chromosomes. Using methods and materials described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have generated transgenic mice by microinjection of 60 Mb murine satellite DNA-based artificial chromosomes containing multiple copies of the *lacZ* ( $\beta$ -galactosidase) and *hph* (hygromycin phosphotransferase) genes into the pronucleus of mouse zygotes.

Fluorescence *in situ* hybridization (FISH) analysis of preimplantation embryos injected with satellite DNA-based artificial chromosomes demonstrated that 44% of the analyzed embryos contained intact satellite DNA-based artificial chromosomes in 8-67% of the total cells analyzed for each positive embryo. In  $\beta$ -galactosidase staining assays of injected preimplantation embryos at various developmental stages, 31% of the analyzed embryos showed X-gal staining indicating the presence of a functional marker gene in the artificial chromosomes

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that was expressed in a mosaic pattern. Seven percent of the mice born after implantation of injected embryos into pseudopregnant female mice were positive for the *hph* gene in nucleic acid amplification analyses of tail DNA. The presence of intact satellite DNA-based artificial chromosomes in mitogen-activated peripheral blood lymphocytes from a female transgenic founder was confirmed by FISH analysis. This analysis also revealed that the artificial chromosomes were maintained as discrete chromosomes in approximately 60% of the cells analyzed and that they had not integrated into the endogenous chromosomes.

Mating of the transgenic female founder with wild-type F1 males yielded progeny, 46% of which were positive for the presence of the *hph* gene in nucleic acid amplification assays of tail DNA. FISH analysis of peripheral blood lymphocytes from progeny carrying the satellite DNA-based artificial chromosomes revealed that intact artificial chromosomes were present in approximately 60% of the analyzed cells with no apparent translocation of the artificial chromosome DNA onto the host chromosomes.

The results of these analyses demonstrate that satellite DNA-based artificial chromosomes as described in the above-referenced application can be used in standard methods of transgenic animal generation to yield viable transgenic animals containing within their cells intact, heterologous gene-containing artificial chromosomes as autonomous, stably replicating, extrachromosomal elements. Furthermore, the results of these analyses demonstrate that the satellite DNA-based artificial chromosomes are transmitted through the germline.

A description of the above-referenced methods, animals and results follows.

**I. Materials and methods**

**A. Satellite DNA-Based Artificial Chromosomes**

Satellite DNA-based artificial chromosomes were obtained from a mouse-hamster-human cell line containing a 50-60 Mb micro-megachromosome

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carrying the anti-HIV *gag* ribozyme and the hygromycin phosphotransferase and  $\beta$ -galactosidase genes. The cell line was generated in accordance with methods described in detail in the above-referenced application.

Specifically, the H1xHe41 cell line (mouse-hamster-human hybrid cell line carrying a megachromosome and a single human chromosome with CD4 and *neo<sup>r</sup>* genes), which is described in the above-captioned application on page 68, lines 9-15, was subjected to repeated BrdU treatment followed by single cell cloning to yield the mM2C1 cell line. As described in the above-referenced application, the H1xHe41 cells are ultimately derived from EC3/7C5 cells that had been co-transfected with pCH110 and pH132. These plasmids carry the  $\beta$ -galactosidase-encoding gene (*lacZ*), which is linked with the SV40 promoter, (pCH110) and the hygromycin-resistance gene (*hph*) and anti-HIV *gag* ribozyme under control of the  $\beta$ -actin promoter (pH132; see pages 53-54 of the above-captioned application for a description of construction of pH132).

The mM2C1 cell line contains the ~60 Mb megachromosome containing the anti-HIV *gag* ribozyme and the *hph* and *lacZ* genes. mM2C1 cells were fused with chinese hamster ovary (CHO) cells by microcell fusion to generated CHO-E4-20 cells as follows. Mitotic cells were harvested from colchicine-treated mM2C1 cells and centrifuged through a percoll gradient in the presence of cytochalasin-B. Microcells were passed through successive filters and overlaid for 20 minutes onto recipient CHO cells and treated with polyethylene glycol. Selection for clones containing satellite DNA-based artificial chromosomes was based on expression of  $\beta$ -galactosidase using hygromycin resistant growth techniques. Single-cell cloning by limiting dilution was repeated several times to yield the CHO-E4-20 cell line that contains two intact, functional ~60 Mb megachromosomes. The cell line was grown under standard conditions in MEM- $\alpha$  medium under selective (0.15  $\mu$ g/ml hygromycin) conditions.

**B. Isolation and Purification of Satellite DNA-Based Artificial Chromosomes**

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The satellite DNA-based artificial chromosomes were purified from CHO-E4-20 cells using flow cytometry generally as described in the above-referenced application. The artificial chromosomes were first isolated from the cells as follows. Cells were plated in 150 mm tissue culture dishes and supplemented with fetal calf serum and hygromycin B. After 24 hours, exponentially growing cells were blocked in mitosis with colchicine (1.0  $\mu\text{g/ml}$ ) for 7 hours before harvest. Mitotic cells collected by washing were swollen in a hypotonic buffer of 75 mM KCl for 10 min at room temperature. After swelling, the cells were transferred to the polyamine buffer (80 mM KCl, 70 mM NaCl, 0.1%  $\beta$ -mercaptoethanol, 15 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 0.2 M spermine, 0.5 M spermidine, and 0.25% Triton X-100, adjusted to pH 7.2) and incubated on ice. Shearing of the cell membranes was achieved by gently drawing the cell suspension up and down a 22-gauge needle attached to a 10-ml syringe. Hexylene glycol (2%)/200mM glycine buffer was added to an equal volume of the polyamine buffer containing the released chromosomes giving a final volume of 20 ml. Prior to staining, the chromosome preparation was centrifuged at 100g for 1 minute to remove cellular debris.

The chromosome suspension (supernatant) was removed to a fresh tube and stained with Hoechst 33258 (2.5  $\mu\text{g/ml}$ ), chromomycin A3 (50  $\mu\text{g/ml}$ ) in the presence of 2.5 mM  $\text{MgCl}_2$ . Samples were stored at 4°C for a minimum of 2 hours. Fifteen minutes before flow cytometric sorting, 10 mM sodium citrate and 25 mM sodium sulfite were added. A final concentration of 15-20 million chromosomes per ml was achieved. All chromosome preparations were filtered through a 35- $\mu\text{m}$  nylon mesh and stored on ice until sorted.

Purification of the satellite DNA-based artificial chromosomes from the chromosome preparation was performed on a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a Turbo-Sort Option and two Innova 306 lasers (Coherent, Palo Alto, CA). Hoechst 33258 was excited with the primary UV laser beam and excitation detected in FL1 using a 420-nm band-pass filter, whereas chromomycin A3 was

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excited by the second laser set at 458 nm, and fluorescence detected in FL 4 by using a 475 nm long-pass filter. The sheath buffer used in the sorting procedure contained 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 30  $\mu$ M spermine and 70  $\mu$ M spermidine. Flow-sorted satellite DNA-based artificial chromosomes were pelleted by centrifugation of a 1-ml sample containing  $\sim 10^6$  chromosomes at 2500 x g for 15 minutes at 4°C, and finally retaining only 10-20  $\mu$ l of loose pellet.

**C. Culture of Murine Embryos**

Mouse zygotes were collected from superovulated (C57BL/6 x CBA) F1 females approximately 12 hr after fertilization. All mice were viral antibody-free and maintained in a barrier facility according to University of British Columbia guidelines. Harvesting, culture, and implantation of embryos were done using standard procedures [see, e.g., Hogan *et al.* (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*. New York: Cold Spring Laboratory Press].

**D. Microinjection of Satellite DNA-Based Artificial Chromosomes**

For murine pronuclear injections, non-filamented borosilicate glass micropipettes (Pyrex, Corning No. 77, 1.0 mm O.D. x 0.75 mm I.D.) were pulled and bevelled using a Sutter Model P-97 micropipette puller and a Sutter Model BV-10D micropipette beveler fitted with a 104F fine diamond plate, respectively. Zygotes were deposited in a 12  $\mu$ l volume of injection medium on a depression microscope slide. The injection medium consisted of one part concentrated satellite DNA-based artificial chromosomes in sheath buffer and 3 parts M2 medium (Sigma), and was covered by mineral oil. Pronuclei and satellite DNA-based artificial chromosomes (approximately 2  $\mu$ m x 1  $\mu$ m) were visualized using Leica differential interference contrast optics. One or more artificial chromosomes were frontloaded into the tip of the needle by applying suction on the microinjection needle using a manual air-driven SAS11/2-E equilibrating syringe (Research Instruments, Cornwall, England), and injected into the male pronucleus.

**E. Genomic DNA Extraction, PCR and  $\beta$ -galactosidase Staining**

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Mouse tail biopsies were obtained under anesthesia at weaning. Total DNA from mouse tails or parts of newborn mice were extracted according to Hogan *et al.* (1994). DNA (400 ng) was amplified using AmpliTaq kit (Perkin Elmer, Foster City, CA). Each 50  $\mu$ l reaction contained 1x PCR Buffer II (Perkin Elmer), 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP, 0.5  $\mu$ M each primer, and 2.5 units of AmpliTaq Gold™ DNA polymerase (Perkin Elmer). The presence of hygromycin phosphotransferase transgene was detected by amplification of an internal 414-bp fragment using the following primers:

5'-CGGGGGCAATGAGATATGAAAAAG-3' and

5'-GAACCCGCTCGTCTGGCTAAG-3'.

The PCR reaction mixtures were heated to 95°C for 10 min. and subjected to 35 cycles of amplification (94°C 1 min., 59.3°C 1 min., 72°C 1 min.), followed by 10 min. at 72°C. Embryos were assayed for  $\beta$ -galactosidase activity according to Takeda and Toyoda [(1991) *Mol. Reprod. Dev.* 30:90-94].

F. Fluorescence in situ Hybridization

Metaphase spreads from murine embryos were made based on the technique of Garside and Hillman [(1985) *Experientia* 41:1183-1184]. Embryos were arrested in M16 media containing 5  $\mu$ g/ml colcemid (Sigma) for about 1 hour or 3  $\mu$ g/ml nocodazole (Sigma) for 12 to 16 hours at 37°C in 5% CO<sub>2</sub>. Metaphase spreads from peripheral blood lymphocytes were done according to standard procedures [Dracopoli *et al.* (1994) *Current Protocols in Human Genetics*, New York: John Wiley & Sons]. 100  $\mu$ l of blood was aseptically collected from each live mouse by saphenous vein bleeding [Hem *et al.* (1998) *Lab. Anim.* 32:364-368], and cultured for three days in a humidified atmosphere at 37°C/5% CO<sub>2</sub> in RPMI 1620 (Gibco/BRL) supplemented with 20% Fetal bovine serum, 3  $\mu$ g/ml concanavalin A (Type IV-S, Sigma), 10  $\mu$ g/ml lipopolysaccharide (Sigma), and 5 x 10<sup>-5</sup>M  $\beta$ -mercaptoethanol (Gibco BRL). Colcemid was added to a concentration of 0.25  $\mu$ g/ml and the culture was further incubated at 37°C/5% CO<sub>2</sub> for 2 hours. Mouse major satellite DNA, *lacZ* or *hph* probes were labelled using the Biotin-Nick Translation Mix

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(Boehringer Mannheim) or the DIG-Nick Translation Mix (Boehringer Mannheim). Fluorescence *in situ* hybridization (FISH) was done as described [Pinkel *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:2934-2938]. At least 100 lymphocytes were scored at 95% confidence levels as calculated by applying binomial statistics.

**II. Analysis of Injected Mouse Embryos**

**A. Survival**

Up to half of all the injected murine embryos survived pronuclear injection of the satellite DNA-based artificial chromosomes (Table 1). Over 50% of the viable artificial chromosome-injected zygotes developed to the blastocyst stage and 47% (14/30) of control buffer-injected embryos reached the blastocyst stage, indicating that the artificial chromosomes did not adversely affect early embryo development.

Table 1. Murine embryo survival and development after pronuclear injection of satellite DNA-based artificial chromosomes

Experiment No.	Viable Embryos/Total Injected	2-Cell Embryos on Day 2 of Culture	Blastocysts on Day 4 of Culture
1	4/31 (31%)	3/4 (75%)	2/4 (50%)
2	8/24 (33%)	4/8 (50%)	4/8 (50%)
3	21/44 (48%)	19/21 (90%)	13/21 (62%)

**B. Presence of Satellite DNA-Based Artificial Chromosomes**

To look for the presence, and to evaluate the fate of injected satellite DNA-based artificial chromosomes, FISH was performed on chromosome spreads of preimplantation embryos injected with artificial chromosomes. Morulae or blastocysts were probed with *lacZ*. The satellite artificial chromosome was present in 44% of murine embryos analyzed. Embryos exhibited varying degrees of mosaicism for the presence of satellite DNA-based artificial chromosomes (Table 2), which was detected in 8% to 67% of cells scored for each positive embryo. Translocation events between the artificial chromosome marker sequences and native chromosomes were not observed,

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and the metaphase artificial chromosomes appeared to be intact at the level of FISH analysis.

Table 2. Transgenesis rates in murine morulae and blastocysts derived from satellite DNA-based artificial chromosome-injected embryos.

No. of Embryos Analyzed	No. of Embryos Containing the Satellite DNA-Based Artificial Chromosome	Developmental Stage of Positive Embryos	No. of Positive Blastomeres/Total Blastomeres Analyzed
18	8 (44%)	Morula	12/25 (48%)
		Blastocyst	4/8 (50%)
		Blastocyst	2/3 (67%)
		Blastocyst	5/33 (15%)
		Blastocyst	2/26 (8%)
		Blastocyst	6/10 (60%)
		Blastocyst	4/22 (18%)
		Blastocyst	9/23 (39%)

**C. Expression of the *lacZ* Gene Contained within the Artificial Chromosomes of Injected Embryos**

Transcriptional activity of *lacZ* on the satellite DNA-based artificial chromosomes was investigated in preimplantation mouse embryos injected with artificial chromosomes. Murine zygotes were injected with one artificial chromosome and analyzed for  $\beta$ -galactosidase activity after 4 days in culture. Nine out of 14 embryos (64%), at various stages of development, showed X-gal staining, indicating the presence of a functional marker gene within the artificial chromosome. All embryos exhibited a mosaic expression pattern.

**III. Analysis of Founder Mice**

Embryos injected with satellite DNA-based artificial chromosomes were implanted into pseudopregnant females. PCR analysis of tail DNA samples revealed that 3 of the 44 mice (7%) born were positive for the *hph* gene (Table 3). One of these was a male that died perinatally (one other PCR-negative pup from the same litter also died perinatally) and the other two (one male, one female) were healthy and phenotypically normal (similar weight/size as non-

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transgenic siblings or other mice of the same age). Mitogen-activated peripheral blood lymphocytes from the female transgenic founder were analyzed by FISH using an *hph* probe. The chromosome spreads revealed the presence of intact artificial chromosomes in approximately 60% of the cells analyzed (Fig. 1). This pattern was seen in 58% of the metaphase spreads observed, each of which also maintained the normal diploid chromosome number of 40. There was no detectable evidence of translocation of artificial chromosome marker sequences onto host chromosomes.

Table 3. PCR analysis of mice derived from satellite DNA-based artificial chromosome-injected embryos.

Experiment No.	No. of Injected Embryos	No. of 2-Cell Embryos Implanted	No. of Mice Born	No. of <i>hph</i> Mice (percentage of positive mice)
1	63	20	8 <sup>a</sup>	1 <sup>b</sup> male (13%)
2	115	20	9	1 female (11%)
3	50	22	6	0
4	78	17	8	0
5	48	19	7	0
6	39	11	6	1 male (17%)
Total	393	109	44	3 (7%)

<sup>a</sup>Two out of eight pups died perinatally.

<sup>b</sup>Died perinatally.

#### IV. Analysis of Founder Progeny

The female transgenic founder was mated with wild type (C57BL/6 x CBA) F1 males. Out of six litters, a total of 41 progeny were born, 19 of which were transgenic (46%) as determined by PCR analysis of tail DNA for the presence of the *hph* gene. Of eleven surviving mice, 6 were transgenic (55%); of the 30 mice that died apparently as a result of maternal neglect, 13 were transgenic (43%), indicating that progeny harboring artificial chromosomes did not have a higher incidence of mortality as compared to progeny without artificial chromosomes. FISH analysis of metaphase spreads of peripheral blood

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lymphocytes from the artificial chromosome-positive progeny revealed the presence of intact satellite DNA-based artificial chromosomes, with no apparent translocation of artificial chromosome marker DNA onto the host chromosomes.

The artificial chromosome was detected in approximately 60% of the lymphocytes analyzed and this level was maintained stably for many months in both the founder and its progeny (Fig. 1). The finding that not all lymphocytes were artificial chromosome-positive may have been due in part to limitations of the FISH technique wherein the occurrence of false negative cells cannot be accurately assessed. However, we have previously demonstrated that we can detect the satellite DNA-based artificial chromosome in up to 96% of metaphase spreads analyzed in cell lines carrying the same artificial chromosome using the same standard technique [Telenius *et al.* (1999) *Chromosome Res.* 7:3-7].

Mosaicism in founder mice is common in conventional transgenesis methods involving the transfer of naked DNA [Chan *et al.* (1999) *Mol. Reprod. Dev.* 52:406-413]. A delay in the onset of satellite DNA-based artificial chromosome replication after microinjection into the pronucleus may account for the mosaicism observed in peripheral blood tissue. A similar delay in the onset of replication of the artificial chromosome during the first round(s) of embryo cell division might also account for the mosaicism observed in the progeny.

Metaphase spreads obtained from the artificial chromosome-negative siblings of both the founder and its progeny were negative for the presence of the artificial chromosomes by FISH analysis. The growth rates and body size of the positive progeny were comparable to those of non-transgenic littermates. To date, the founder mouse and her six transgenic progeny have not exhibited any overt phenotypic abnormalities.

#### V. Summary and Conclusions

Using satellite DNA-based artificial chromosomes and methods described in the above-referenced application, it has been possible to generate viable transgenic mice that stably maintain satellite DNA-based artificial chromosomes as discrete elements that remain separate from the host genome. The artificial

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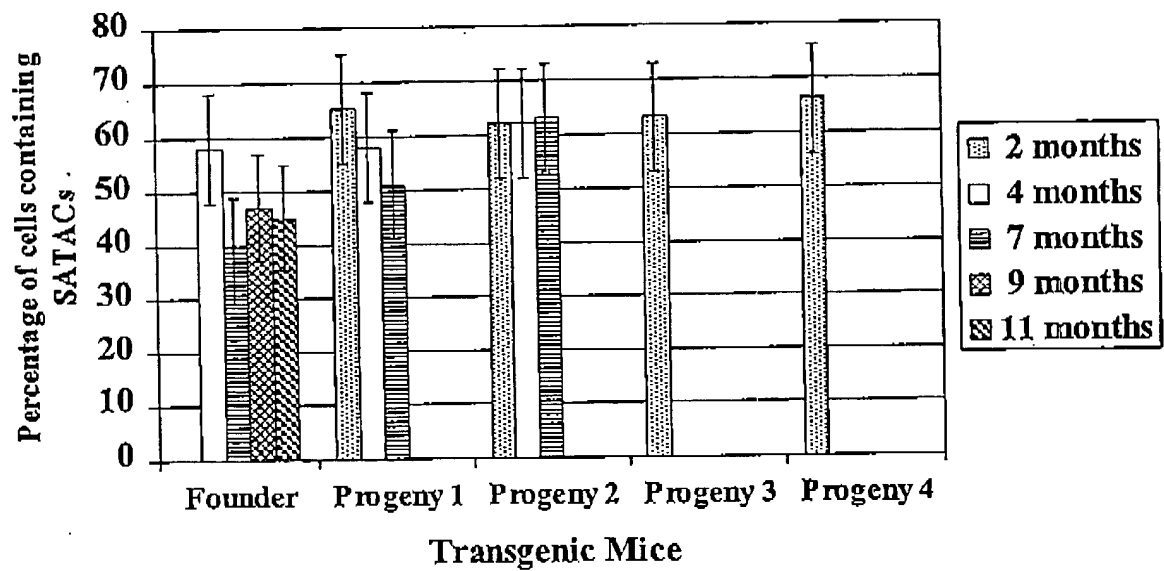
chromosomes were able to undergo mitotic segregation from early embryonic development in the absence of selective pressure, and did not show evidence of integration into the host genome. The maintenance of the satellite DNA-based artificial chromosomes within cells was sustained into adulthood in the mice, and importantly, these chromosomes were transmitted through the germline, as demonstrated by the presence of these artificial chromosomes in 50% of the founder progeny.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

\_\_\_\_\_  
**Carl Perez**

Date: \_\_\_\_\_

Figure 1



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Paula - You're right. I only faxed you the 1st page article

# Functional expression and germline transmission of a human chromosome fragment in chimaeric mice

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Human chromosomes or chromosome fragments derived from normal fibroblasts were introduced into mouse embryonic stem (ES) cells via microcell-mediated chromosome transfer (MMCT) and viable chimaeric mice were produced from them. Transferred chromosomes were stably retained, and human genes, including immunoglobulin (Ig)  $\kappa$ , heavy,  $\lambda$  genes, were expressed in proper tissue-specific manner in adult chimaeric tissues. In the case of a human chromosome (hChr.) 2-derived fragment, it was found to be transmitted to the offspring through the germline. Our study demonstrates that MMCT allows for introduction of very large amounts of foreign genetic material into mice. This novel procedure will facilitate the functional analyses of human genomes *in vivo*.

Various techniques for producing chimaeras from genetically manipulated mouse ES cells have provided valuable tools for studying gene functions *in vivo* and developmental regulation of gene expression. A variety of procedures such as targeted modification of specific genes by homologous recombination<sup>1</sup>, introduction of cloned exogenous DNA fragments<sup>2,3</sup> and chromosome manipulation by the Cre-loxP system<sup>4,5</sup> have facilitated the genetic manipulation of ES cells. Cell fusion, or chromosome transfer, is an indispensable tool for characterizing gene expression in somatic cells<sup>6</sup>. Although several such studies using embryonic carcinoma (EC) cells<sup>7</sup> have been reported, these techniques have rarely been used to manipulate ES cells. The creation of specified foreign-chromosome function in animals through ES cells would enable the study of very large genes or gene clusters *in vivo*, beyond the limited length of DNA that can be cloned by conventional techniques and reproduce proper expression of introduced genes by inclusion of critical remote regulatory elements. Moreover, it will be possible to construct model animals representing human genetic diseases involving chromosome abnormalities.

Nineteen years ago, Illmensee and co-workers reported the production of viable chimaeric mice from hybrid mouse EC cells fused with human fibrosarcoma or rat hepatoma cells<sup>8,9</sup>. However, they could not unequivocally demonstrate that the hybrid EC cells had contributed to normal tissues of the mice. It has generally been supposed that extensive genetic modification such as cell fusion or chromosome transfer causes loss of pluripotency, therefore this issue has never been examined seriously.

In this study, we have systematically developed a means by which to produce viable chimaeric mice from microcell-hybrid ES (MH(ES)) cells containing a transferred human chromosome originally derived from normal fibroblasts. We have investigated: (i) the pluripotency of MH(ES) cell lines, (ii) the viability of chimaeric mice derived from MH(ES) cell lines with artificial aneu-

ploidy, (iii) the retention of transferred chromosomes in adult chimaeric tissues, (iv) expression of human genes in various chimaeric tissues and (v) germline transmission of transferred chromosomes to the offspring.

We report a novel procedure to introduce foreign genetic material into mice by using the chromosome itself as a 'vector'. Human chromosome (hChr.) 14-, 22- or 2-derived fragments, which include Ig heavy,  $\lambda$ , or  $\kappa$  genes, respectively, could be transferred into mouse ES cells via MMCT<sup>10</sup>. Their contribution to a variety of normal chimaeric tissues in viable chimaeras revealed the pluripotency of resultant MH(ES) cells — several human genes harboured by the transferred chromosomes were expressed in a normal tissue-specific manner. For example, we documented the expression of each human Ig polypeptide in the sera of the chimaeric mice and a diversity of V segments in the variable regions of rearranged human Ig transcripts. Finally, we have determined that male and female chimaeras containing a hChr.2-derived fragment could transmit this fragment to their offspring via the germline.

## Construction of MH(ES) cells

Our strategy to introduce human chromosomes into mice is outlined in Fig. 1. We utilized MMCT to introduce a specific human chromosome into mouse ES cells. For this purpose, we constructed a new library of human-mouse A9 monochromosomal hybrids containing human chromosomes derived from normal embryonic fibroblasts. The library comprises approximately 700 independent hybrid clones, each of which contains a human chromosome, randomly tagged with pSTneoB<sup>11</sup> suitable for conferring G418-resistance to mouse ES cells. We used these as a source of microcell donor cell lines for MMCT into mouse ES cells. Our primary goal was to reconstitute the repertoire of human antibodies in mice from unrearranged human Ig genes, of which total sizes are more than 1.0–2.0 Mb on hChr.2, 14 and 22 (reviewed in ref.12). There-

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## article

fore, we screened these hybrid clones with PCR primers specific to human Ig  $\kappa$ , heavy,  $\lambda$  genes and markers on hChr.2, 14 or 22 in order to obtain the clones retaining at least the human Ig locus. We thus chose four hybrid A9 clones as donors for MMCT (Table 1a). We further analysed microcell-hybrid A9 clones A9/2-W23, A9/14-C11 and A9/22-G2 by Southern-blot hybridization using a human-specific L1 repeat sequence as a probe (data not shown) and observed specific L1 banding patterns for each hybrid A9 clone. We identified an independent human chromosome or its fragment in each hybrid by fluorescence *in situ* hybridization (FISH, Table 1a). Hybrid clones A9/2-W23 and A9/22-G2 contained a small fragment (referred to as hCF(2-W23); Fig. 2a), retaining a small number of hChr.2 markers adjacent to the centromeric region. The *Alu*-PCR probe<sup>13,14</sup> generated from these hybrids exclusively hybridized to the centromeric region of hChr.2 on normal human metaphase samples (data not shown), suggesting that the hCF(2-W23) contained part or all of the human centromere region. Intact human chromosomes 14 and 22 were identified in A9/14-C11 and A9/22-G2, respectively.

Mouse ES cells (TT2 (ref. 15): 40,XY or TT2F (ref. 16): 39,XO) were fused with the microcells prepared from these donor hybrid

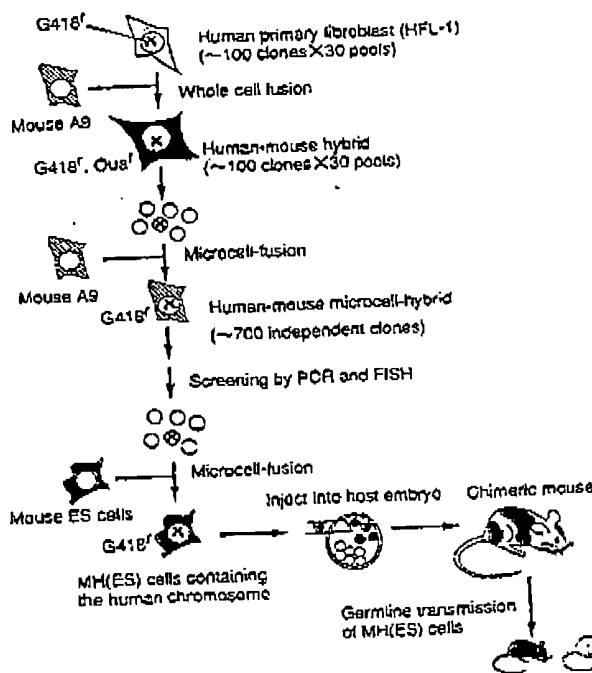


Fig. 1 A schematic diagram showing the construction of MH(ES) cells to produce chimeric mice expressing human genes on the transferred chromosomes. The construction and analysis of the library containing approximately 700 clones of microcell-hybrids will be described elsewhere in detail (HLL and M.O., manuscript in preparation). G418<sup>r</sup>: G418-resistant; Ouf<sup>r</sup>: ouabain-resistant.

MH(ES)22-1 (Fig. 1a,c) were similar to those observed in donor cell lines (data not shown). MH(ES)2-1 was also probed with the sequence for human Ig  $\kappa$  gene (2q12) to confirm its location on the hCF(2-W23) (Fig. 2d). Various morphological alterations of the hChr.14 were observed in four MH(ES) clones from A9/14-C11

(one of the four clones. MH(ES)14-4 is shown in Fig. 2b), suggesting chromosomal rearrangements. Chromosome numbers of MH(ES)2-1 and MH(ES)14-4 revealed that more than 90% of the spreads contained 41 chromosomes consisting of 40 normal mouse chromosomes and an additional human-derived chromosome fragment (Fig. 2a,d). We observed

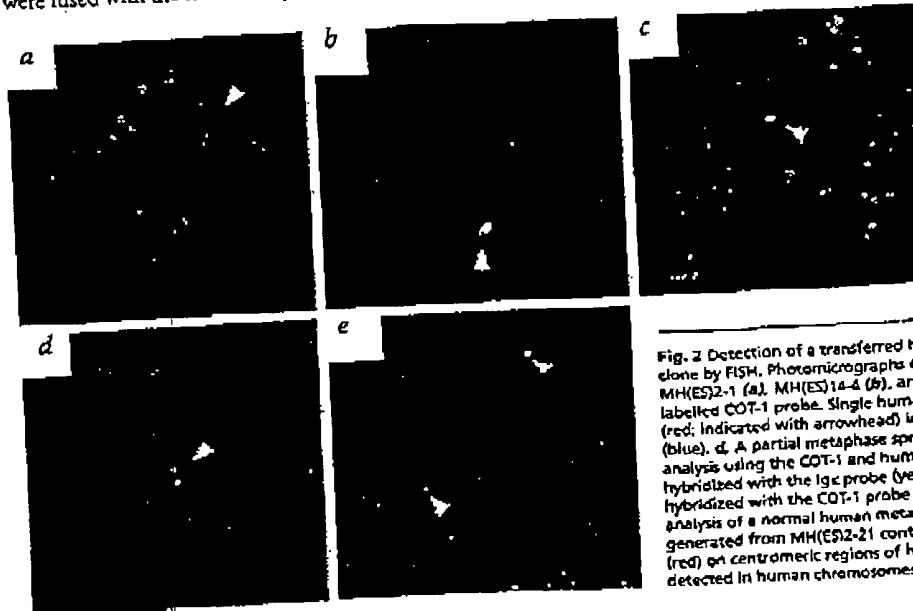


Fig. 2 Detection of a transferred human chromosome in each MH(ES) clone by FISH. Photomicrographs of representative metaphase spreads of clones by FISH. (a) MH(ES)2-1, (b) MH(ES)14-4, (c) MH(ES)22-1, (d) MH(ES)2-1 hybridized with COT-1 probe. Single human-derived chromosomes were detected (red; indicated with arrowhead) in addition to mouse chromosomes (blue). (e) A partial metaphase spread from MH(ES)2-1 by two-colour FISH analysis using the COT-1 and human Igk probes. One pair of dots hybridized with the Igk probe (yellow) is detected on the hCF(2-W23) hybridized with the COT-1 probe (red; indicated with arrowhead). (e) FISH hybridization of a normal human metaphase spread with the *Alu*-PCR probe. Hybridization signals generated from MH(ES)2-1 containing hCF(2-W23). Hybridization signals (red) on centromeric regions of hChr.2 (indicated with arrowhead) were detected in human chromosomes (blue).

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Table 1 - Summary of characteristics of hybrid cell lines obtained and used in this work

a. microcell-hybrid A9 clones													
Hybrid A9 clones	Human chromosomes identified in hybrids	Selectable markers on human chromosomes	Human DNA markers identified in hybrids by PCR analysis <sup>b</sup>										
			hChr2	S171	S134	CD8A	IGKC	IGKV1	FA8P1	S438	S113	S159	
A9/2-W23	hChr2-W23	neo <sup>r</sup>	TPO	-	-	-	-	+	+	+	+	-	
A9/2-W23*	hChr2-W23	neo <sup>r</sup> +puro <sup>r</sup>	-	-	-	-	-	+	+	+	+	-	
A9/14-C11	intact 14	neo <sup>r</sup>	hChr14	NP	MYH6	S75	S66	S43	PC1	S78	IGHMC	IGHV3	
			TCRA	+	+	+	+	+	+	+	+	+	
A9/22-G2*	intact 22	neo <sup>r</sup> +puro <sup>r</sup>	hChr22	MB	PVALB	IL2LB	ARSA	DIA1					
			IGLC	+	+	+	+	+	+	+	+	+	
b. MH(ES) cell lines													
MH(ES) transmission <sup>b</sup> cell lines	Microcell donors	Recipient ES cell lines	MH(ES) selection	Chromosome number	Human chromosomes observed in MH(ES) cells	Human DNA markers identified in hybrids by PCR analysis <sup>c</sup>					Chimera production <sup>e</sup> (five born chimerae / transferred embryos)	Germ-line (germline chimerae / test-bred chimerae)	
TAH(ES)2-1	A9/2-W23	TT2	G418	40+1	hChr2-W23 (see Fig. 2a)	IGKC, IGKV1, FA8P1, D2S1331, D2S338, D2S113						22(10)/404	4/8
MH(ES)14-4	A9/14-C11	TT2	G418	40+1	rearranged hChr.14 <sup>d</sup> (see Fig. 2b)	TCRA, NP, MYH6, S75, S78, IGHMC, IGHV3						5(4)/221	0/1 <sup>b</sup>
MH(ES)14-5	A9/14-C11	TT2	G418	n.d.	rearranged hChr.14 <sup>d</sup>	all 10 tested markers						2(11)/110	0/2
MH(ES)14-6 <sup>f</sup>	A9/14-C11 <sup>c</sup>	TT2	G418	n.d.	rearranged hChr.14 <sup>d</sup>	9 markers except PC1						2(0)/103	n.d.
MH(ES)14-7 <sup>f</sup>	A9/14-C11 <sup>c</sup>	TT2	G418	n.d.	two different fragments <sup>d</sup>	S75, PC1, S78, IGHMC, IGHV3						9(4)/467	0/3 <sup>b</sup>
MH(ES)22-1	A9/22-G2	TT2	puromycin	39+1	intact hChr22 (see Fig. 2c)	all 6 tested markers						8(3)/266	0/1
MH(ES)2-21	A9/2-W23P	TT2F (39.XO)	puromycin	39+1	hChr2-W23 (see Fig. 2c)	IGKC, IGKV1, FA8P1, D2S1331, D2S338, D2S113						10(8)/441	4/5

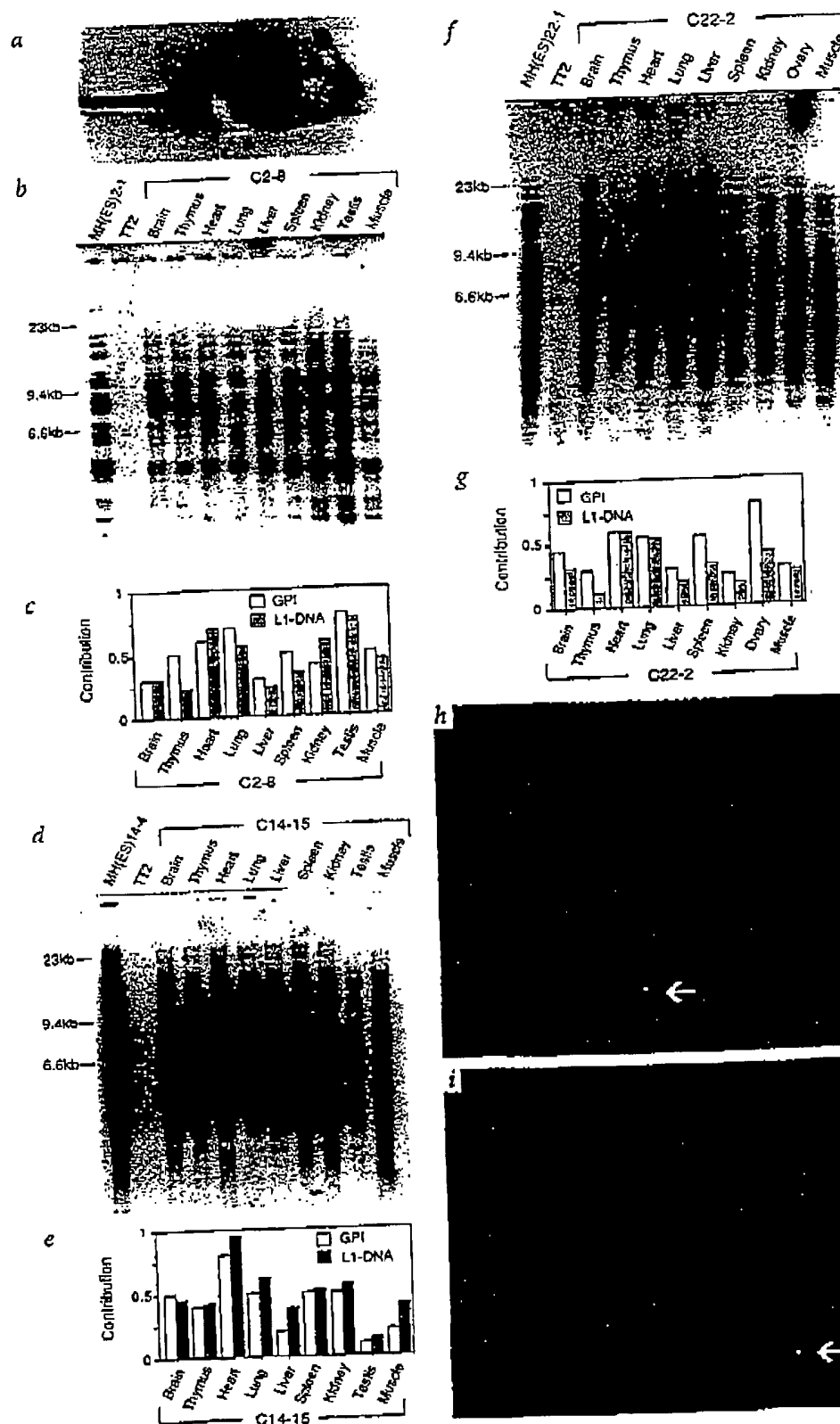
a, A hChr2-W23 or hChr22 is doubly tagged with pStNeoB and pStGuro (ref. 51) in each hybrid A9 clone (K.C. and L.L. manuscript in preparation). b, The approximate orders of marker loci on each chromosome are shown. For example, the relative location of the five tightly linked polymorphic markers (D2S438-S373) to the three loci (IGKC, IGKV1, FA8P1) has not been determined. Symbols are as follows: +, presence; -, absence; c, Gamma-irradiated (30 Gy) microcells were used for the hChr2 in order to introduce hChr14 containing deletions. d, Various morphological alterations of hChr14 were observed in Q-banded metaphase spreads prepared from these MH(ES) clones, although precise assessment of the structural abnormalities of the rearranged hChr14 has not been performed in the present study. e, MH(ES) clones were examined by PCR analysis with the markers for each human chromosome listed in Table 1a. The markers present in each MH(ES) clone are shown. f, The numbers of the chimerae with >50% apical coat are given in parentheses. g, Male chimerae produced from TT2-derived MH(ES) lines and female chimerae from MH(ES)2-21 were used for the breeding. In the case of MH(ES)22-1 lacking a Y chromosome, a recipient female chimera was tested. Details of breeding data from germline chimerae are shown in Table 3. h, These tested male chimerae showed sterility associated with small testes. hChr, human chromosome; hChr2-W23, a human chromosome fragment derived from centromeric region of hChr2; n.d., not determined.

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**Fig. 3** Retention of a transferred human chromosome in chimaeric mice. **a**, A photograph of a representative viable male chimaera produced from MH(ES)144 (C14-15, 10 week old). **b**, **d**, **f**, Southern blots of *Bgl*II-digested genomic DNA (1.5  $\mu$ g) prepared from nine different tissues of viable adult chimaeras (**b**, C2-8, **d**, C14-15, **f**, C22-2) with the L1 probe. DNA sampled from each MH(ES) clone (**b**, MH(ES)2-1, **d**, MH(ES)14-4, **f**, MH(ES)22-1) and TT2 were included as positive and negative controls, respectively. Sizes of *Hind*III-digested DNA markers are shown in kilobases. **c**, **e**, **g**, Quantitative analysis of the retention of human chromosomes in various tissues of adult chimaeras (**c**, C2-8, **e**, C14-15, **g**, C22-2). The contribution of MH(ES)-derived cells to various tissues was determined by the GPI assay (open bar). The relative ratio of radioactivity measured by dot blot hybridization of DNA as the GPI assay using the L1 probe to that of DNA from the injected MH(ES) clones was also determined (closed bar). **h**, **L**, Detection of an independent human chromosome in somatic cells from the chimaeras. Photomicrographs of metaphase spreads of primary fibroblasts prepared from tail of chimaeras (**h**, C14-15, **L**, C22-2) hybridized with the COT-1 probe (red; indicated with arrowheads; see also Fig. 2d,e).

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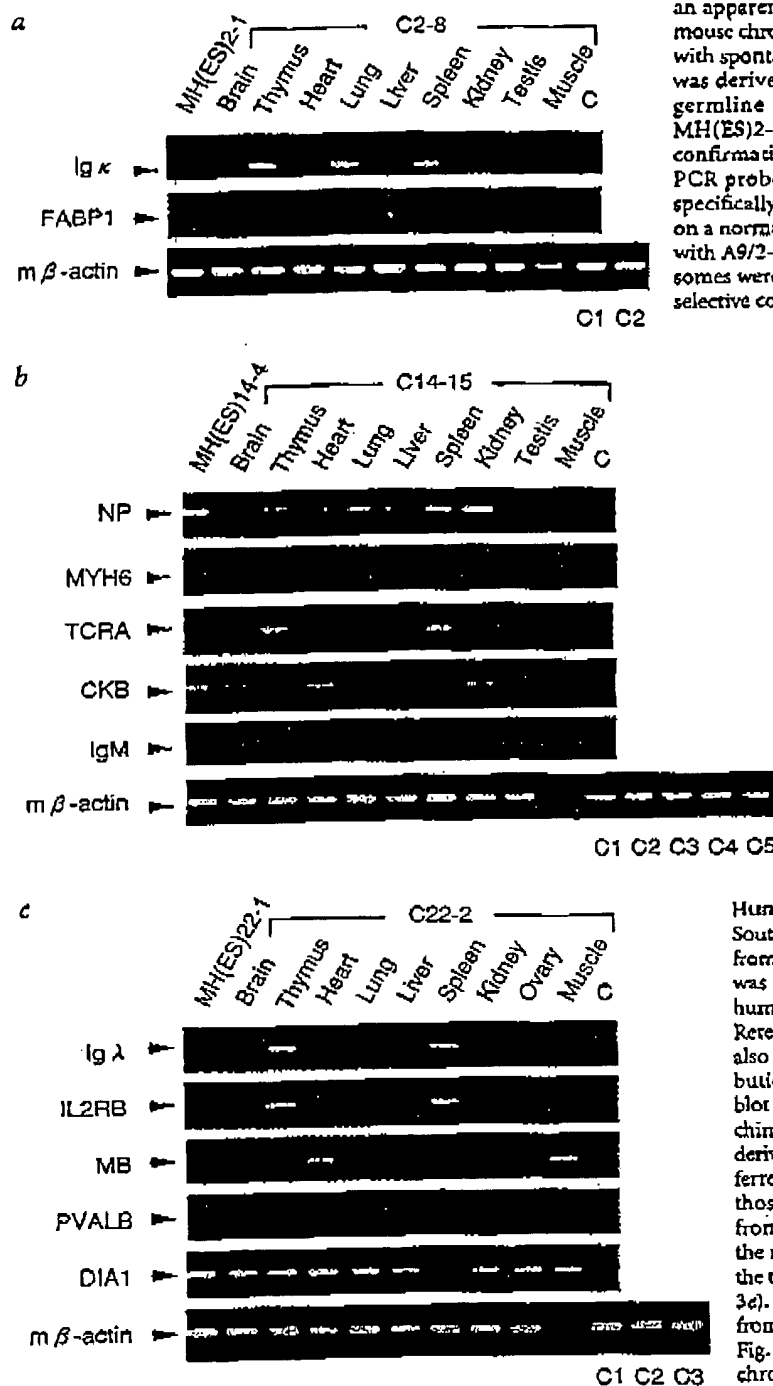


Fig. 4 Detection of human transcripts in various tissues of chimaeras. RT-PCR analysis of total RNA from MH(ES) clones (a, MH(ES)2-1, b, MH(ES)14-4, c, MH(ES)22-1) and nine different tissues from C2-8 (a), C14-15 (b) or C22-2 (c) using human-specific primers. RNA from tissues of non-chimaeric MCH(1CR) mice were used as negative controls (a, C1, spleen for Igk; C2, liver for FABP1; b, C1, liver for NP; C2, heart for MYH6; C3, thymus for TCRA; C4, brain for CKB; C5, spleen for IgM; c, C1, spleen for Igλ and IL2RB; C2, skeletal muscle for MB and PVALB; C3, liver for DIA1). The sizes of resulting PCR products (indicated by arrowheads) in all experiments were consistent with the expected sizes deduced from reported cDNA sequences; murine β-actin was used as a control.

an apparently intact hChr.22 in MH(ES)22-1 alongside 39 mouse chromosomes (in 19 of 20 metaphase spreads; Fig. 2c), with spontaneous loss of the Y chromosome<sup>16</sup>. MH(ES)2-21 was derived from XO ES cells, TT2F, from which female germline chimaeras could be produced efficiently<sup>16</sup>. MH(ES)2-21 retained the hCF(2-W23) (Table 1b). Further confirmation was obtained by FISH analysis with the *Alu*-PCR probe generated from the MH(ES)2-21. The probe specifically hybridized to the centromeric region of hChr.2 on a normal human metaphase sample (Fig. 2c) as observed with A9/2-W23P (data not shown). These human chromosomes were stably maintained in each MH(ES) clone under selective conditions and cell morphology and growth rate of all MH(ES) clones were similar to those of parental TT2 or TT2F cells (data not shown).

#### Chimaera production

We injected the MH(ES) cells into 8-cell stage embryos of albino MCH(1CR) mice and transferred the embryos into the uteri or oviducts of pseudopregnant mice to examine their pluripotency. The result of chimaera production by each MH(ES) clone is shown in Table 1b. Judging from mouse coat colour, all tested MH(ES) clones had the ability to contribute to viable chimaeras (Fig. 3a). Glucose phosphate isomerase (GPI) isozyme assay of various tissues from some chimaeric individuals (see Fig. 3c,e,g) showed that the MH(ES)-derived cells contributed to a substantial fraction of all of the tissues tested, sometimes exceeding 50% contribution. Almost all chimaeras had no recognizable physical abnormalities except that male individuals derived from MH(ES)14-4 and MH(ES)14-7y were sterile, with small testes (see Table 1b).

#### Human chromosomes in chimaeric mice

Southern blot hybridization of genomic DNA prepared from various tissues of the chimaeras with the L1 probe was carried out to assess the retention of transferred human chromosomes in adult chimaeras (Fig. 3b,d,f). Retention of human chromosomes in each tissue was also estimated quantitatively by comparing the contribution determined by the GPI-assay with that by dot blot hybridization with the L1 probe (Fig. 3c,e,g). In the chimaera C14-15 (male, 50% agouti coat, see Fig. 3a), derived from MH(ES)14-4, all tissues retained the transferred human chromosome, with L1 patterns similar to those of the MH(ES)14-4 (Fig. 3d). Results obtained from both the GPI-assay and dot blotting imply that the majority of MH(ES)-derived somatic cells contain the transferred human chromosome in every tissue (Fig. 3e). In C2-8 (male, 70% agouti coat, Fig. 3b,e) derived from MH(ES)2-1, and C22-2 (female, 50% agouti coat, Fig. 3f,g) derived from MH(ES)22-1, each transferred chromosome is also retained in all of the tissues. The significant reduction of the contribution determined by the dot blotting compared to that by the GPI-assay in some tissues (thymus in C2-8, thymus and ovary in C22-2) may indicate the loss of the transferred chromosomes (Fig. 3c,g). All the tissues tested appeared to be normal and no tumor was observed in these chimaeras.

We also confirmed the presence of transferred human chromosomes in somatic cells by FISH analysis of tail

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primary fibroblasts cultured from chimaeras C14-15 and C22-2. A single independent chromosome was hybridized with the COT-1 probe, and its size and morphology found to be the same as that observed in MH(ES)14-4 and MH(ES)22-1, respectively (Fig. 3*b,c*). We also detected an independent chromosome fragment by COT-1 painting in tail fibroblasts of the chimaera produced from MH(ES) cells containing the hCF(2-W23) (data not shown, see also Fig. 5*c*). These results clearly demonstrate that the MH(ES) cells, whose karyotypes are artificially modified by introducing human chromosomes, are able to contribute to various normal chimaeric tissues retaining the human chromosomes.

## Human transcripts in chimaeric mice

To determine whether human genes on the transferred chromosomes were expressed, total RNAs from the MH(ES) cells and various tissues of the chimaeras were analysed by RT-PCR using human-specific oligonucleotide primers for detection of human transcripts (Fig. 4*a,b,c*). Transcripts of nucleoside phosphorylase<sup>18</sup> (NP) and cytochrome b5 reductase<sup>19</sup> (DIA1) were ubiquitously detected in the MH(ES) cells and all tissues examined. Alpha cardiac muscle myosin heavy chain<sup>20</sup> (MYH6) and parvalbumin<sup>21</sup> (PVALB) were detected only in heart and skeletal muscle, respectively, while myoglobin<sup>22</sup> (MB) was expressed in both of the tissues; expression patterns reflecting their unique muscle specificities. FABP1 transcripts, encoding liver fatty acid binding protein<sup>23</sup> were detected mainly in liver as expected. Transcripts of CKB, encoding brain-type creatine kinase, were abundant in the MH(ES) cells, brain, heart and kidney, which is similar to the tissue-specific distribution reported previously<sup>24</sup>. Transcripts of Ig  $\mu$ , Ig  $\kappa$ , Ig  $\lambda$ , T-cell receptor  $\alpha$  (TCRA) and IL-2 receptor  $\beta$  chain<sup>25</sup> (IL2RB) were found mainly in thymus and spleen. Taken together, these findings demonstrate that human genes on the transferred chromosomes derived from differentiated fibroblasts are expressed under proper tissue-specific transcriptional regulation in chimaeric mice throughout their ontogeny.

## Diverse usage of V segments in human Ig transcripts

To confirm the human sequences and to analyse the functional repertoire of human Ig variable regions in the chimaeras, RT-PCR products of human Ig  $\mu$  (h $\mu$ ), Ig  $\kappa$  (h $\kappa$ ) and Ig  $\lambda$  (h $\lambda$ ) genes, including each variable region, were cloned into plasmid vectors and sequenced. Almost all clones contained functional human sequences composed of rearranged human V, J and D segments. The assignment of these sequences to known germline VH, V $\kappa$ , and VA segments revealed strikingly diverse usage which coincides well with that observed in natural human antibodies<sup>26</sup> (Table 2). Predicted amino-acid sequences from CDR3 regions of the cDNA sequence also demonstrate their diversity. This is consistent with previously reported results in YAC transgenic mice<sup>27</sup> with h $\mu$  and h $\kappa$  (data not shown).

Table 3 • Breeding data from germline chimaeras produced from MH(ES) cell lines containing hCF(2-W23)

MH(ES) cell lines	Chimaeras	Total born	Number of F1 offspring	
			Agouti coat color (% of total born)	hCF(2-W23)-positive (% of agouti coat colour)
MH(ES)2-1 (male)	C2-1	37	12(32)	0
	C2-2	103	7(7)	0
	C2-13	53	1(2)	0
	C2-18	123	17(14)	2(12)
MH(ES)2-21 (female)	C2-1F	19	19(100)	8(42)
	C2-2F	5	5(100)	3(60)
	C2-3F	20	20(100)	6(30)
	C2-4F	23	23(100)	5(22)

Table 2 • V segments identified in human Ig transcripts

Chimaeras	V families	Germline V segments
C14-15 (h $\mu$ )	VH1	1-6, 1-18, 1-24, 1-46 (2)
	VH2	2-5
	VH3	3-21, 3-23 (4), 3-33 (3)
	VH4	4-34, 4-59 (2)
C2-8 (h $\kappa$ )	V $\kappa$ 1	O18-O8 (3), L1
	V $\kappa$ 2	A17 (2)
	V $\kappa$ 3	A27 (4)
	V $\kappa$ 6	A26-A10
C22-2 (h $\lambda$ )	VA1	DPL-3, -5 (3), -8
	VA2	DPL-10, -11 (3), -12 (2)
	VA3	DPL-16

Seventeen h $\mu$ , 11 h $\kappa$ , and 12 h $\lambda$  clones which contained unique and functional in-frame sequences were assigned to germline VH, V $\kappa$ , and VA segments reported previously (numbered according to ref. 33, 41 and 49, respectively), and classified into their families. The numbers of independent clones containing the same V segment are shown in parentheses.

## Antibodies with human Igs in chimaeric mice

In addition to detecting and sequencing human Ig transcripts, we assessed their expression in the sera of the chimaeras. Each human Ig in sera of non-immunized chimaeras was identified by ELISA, using anti-human Ig antibodies. h $\mu$  (0.3–7.1 mg/l) and human Ig  $\gamma$  (h $\gamma$ ; 0.3–4.8 mg/l) polypeptides were detected in the sera of all 14 tested chimaeras produced from MH(ES)14-4, 14-5, 14-6 $\gamma$  or 14-7 $\gamma$ . Further analyses revealed the production of all four h $\gamma$  subclasses ( $\gamma$ 1, 2.3;  $\gamma$ 2, 2.0;  $\gamma$ 3, 0.2 and  $\gamma$ 4, 0.4 mg/l) in the chimaera C14-15. h $\kappa$  (2–156 mg/l) polypeptides were detected in 19 of 23 tested chimaeras produced from MH(ES)2-1 or MH(ES)2-21. h $\lambda$  (12–32 mg/l) was also detected in each of the six tested chimaeras produced from MH(ES)22-1.

Despite the fact that the B-cell population containing the human chromosome was less than 100% of the total B-cell population in the chimaeras, the serum concentrations of human Igs were several fold higher than those of YAC transgenic mice<sup>27</sup> (h $\mu$ , 0.9 mg/l; h $\kappa$ , 30 mg/l, the highest concentration in single copy YAC transgenics<sup>27</sup>; h $\mu$ , 7.1 mg/l; h $\kappa$ , 156 mg/l, the highest concentration in our chimaeric mice). Upon immunization of these chimaeras with human serum albumin (HSA), HSA-specific antibodies with h $\gamma$ , h $\kappa$  and h $\lambda$  were readily detected in their sera (data not shown), which is consistent with the result recently reported by Mendez *et al.*<sup>28</sup>. Although the analysis of the integrity of transferred human Ig genes in the B cells of the chimaeras has not yet been performed, these results suggest that human Ig sequences on the transferred chromosomes contain all the elements necessary to reconstitute the diverse and functional repertoire of human Igs observed in humans.

## Germline transmission of a hChr.2-derived chromosome fragment

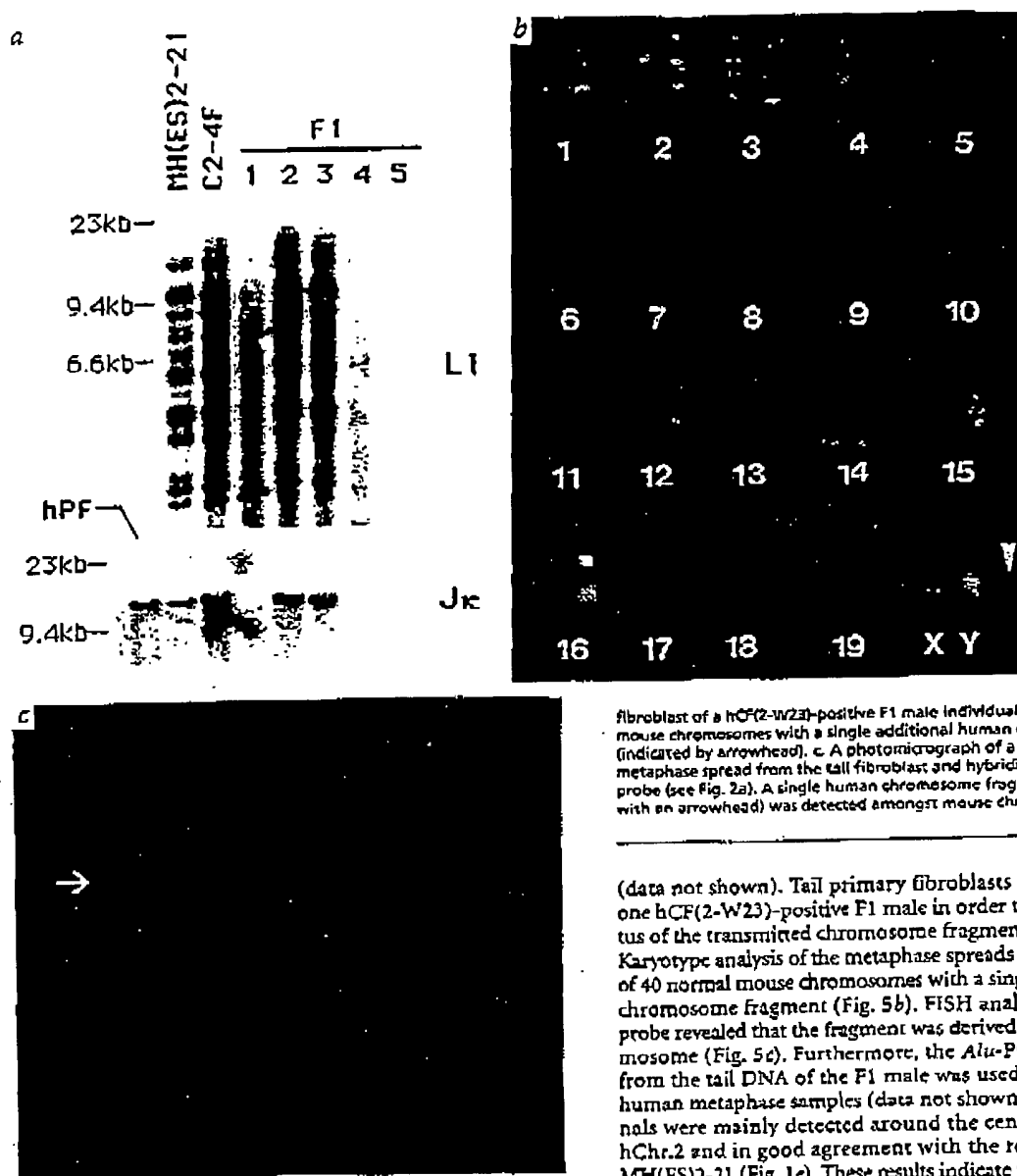
To test whether the MH(ES) cells contribute to the germline, we mated the chimaeras with albino MCH(ICR) mice. So far, we have never observed the birth of dominant-agouti offspring from the chimaeras produced from MH(ES) cell lines containing hChr.14-derivatives or hChr.22 (Table 1*b*). On the other hand, agouti offspring were born from four male chimaeras produced from MH(ES)2-1 and four female chimaeras from MH(ES)2-21 at a frequency of 2–32% and 100%, respectively (Table 1*b*, Table 3). The PCR analysis of genomic DNA prepared from tail of the resultant agouti offspring showed that some of the indi-

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**Fig. 5** Germline transmission of a hChr2-derived fragment. **a**, Southern blots of 8gIII-digested genomic DNA (1.5  $\mu$ g) prepared from the tail of five agouti offspring born from female chimera C2-4F. Hybridization signals with the L1 (top) or Jk (bottom) probes were detected in two of five F1 individuals, indicating the transmission of hCF(2-W23) to F1 offspring through germline. DNA samples from MH(ES)2-21, tail of the mother chimera and human primary fibroblasts (hPF; for the Jk probe) were used as controls. The size of HindIII-digested lambda DNA are indicated. **b**, Karyogram constructed from a photomicrograph of a representative Q-banded metaphase spread prepared from tail

fibroblast of a hCF(2-W23)-positive F1 male individual that showed 40 normal mouse chromosomes with a single additional human chromosome fragment (indicated by arrowhead). **c**, A photomicrograph of a representative metaphase spread from the tail fibroblast and hybridized with the COT-1 probe (see Fig. 2a). A single human chromosome fragment (red; indicated with an arrowhead) was detected amongst mouse chromosomes (blue).

viduals retained all six markers mapped on the hCF(2-W23) (Table 3, hCF(2-W23)-positive), implying that the transferred univalent foreign chromosome could pass through meiosis in both male and female to generate functional gametes. Furthermore, hC polypeptides were detected in the sera of all the hCF(2-W23)-positive F1 individuals at comparable levels (12–316 mg/l) to the parent chimera. They appeared to be normal and we could not distinguish them from negative littermates by their external appearance.

In addition, we sought to elucidate the structural integrity of hCF(2-W23) in the somatic cells of F1 mice. Tail DNAs prepared from five agouti offspring born from a female chimera, C2-4F, were further analysed by Southern blotting with the L1 probe and the probe specific for the J region of Igk gene (Jk; Fig. 5a). Specific bands, observed in MH(ES)2-21 and the mother chimera with these probes, were also observed in two of five offspring. This is consistent with the results of PCR analysis and hC production

(data not shown). Tail primary fibroblasts were prepared from one hCF(2-W23)-positive F1 male in order to investigate the status of the transmitted chromosome fragment in its somatic cells. Karyotype analysis of the metaphase spreads showed the presence of 40 normal mouse chromosomes with a single additional human chromosome fragment (Fig. 5b). FISH analysis with the COT-1 probe revealed that the fragment was derived from a human chromosome (Fig. 5c). Furthermore, the Alu-PCR probe generated from the tail DNA of the F1 male was used in FISH analysis of human metaphase samples (data not shown). Hybridization signals were mainly detected around the centromeric regions of hChr2 and in good agreement with the result obtained from MH(ES)2-21 (Fig. 1e). These results indicate that the overall structure of the hCF(2-W23) has not changed after passage through meiosis. Additionally, we have observed the transmission of hCF(2-W23) down to the F4 generation (details of extensive breeding analysis will be described elsewhere).

#### Discussion

We have clearly demonstrated that a human chromosome or its fragment, derived from fibroblast cells, can function in chimaeric mice produced from MH(ES) cells. It is, therefore, presumed that over one thousand human genes on the transferred chromosome can be expressed under normal tissue-specific regulation in various somatic tissues of the chimera. Although we were initially concerned that it might prove difficult to produce viable chimeras from ES cells with an artificially modified karyotype, it transpires that this is not a serious problem. Thus, genetic modification of mouse ES cells by introduction of human chromosomes

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can be made without causing loss of their pluripotency. Furthermore, this study has provided evidence for germline transmission of a hChr.2-derived fragment. We believe this is the first demonstration of the generation of a novel mouse strain containing a heritable foreign chromosome.

Generation of chimaeric mice retaining a human chromosome. After injection into embryos, the MH(ES) cells proliferate and differentiate during mouse ontogeny without selective force for the retention of the extra human chromosome. Can transferred foreign chromosomes continue to be retained in such an environment? Our results indicate that they are retained in a substantial proportion of the MH(ES)-derived cells in most of the chimaeric tissues tested (Fig. 3); loss was suggested in only a few cases (see Fig. 3c,g). A decrease in cell population retaining the transferred human chromosome with aging might be expected in populations with a rapid turn-over rate such as antibody-secreting B-lymphocytes. However, there was no significant reduction in the serum concentration of human Igs for one year after birth (data not shown). Thus, it is presumed that the transferred human chromosomes, which are maintained as a single independent chromosome in the cells of adult chimaeric tissues (Fig. 3h,i), can be retained in various tissues of the chimerae throughout their life span.

Resultant viable chimerae were physically normal except for some individuals containing hChr.14-derivatives. In spite of the early lethality observed in trisomic mouse embryos<sup>28</sup>, viable aneuploid chimerae could be produced from trisomy (Ts)17 embryos and Ts6 EC cells by chimera formation with normal embryos<sup>30,31</sup>. Our results indicate that this is likely to be the case in 'artificial-aneuploid' MH(ES) cells. Nevertheless, this procedure will enable us to generate mice with aneuploidy or mosaicism containing desired human chromosome or fragments derived from specific chromosomal regions. Such chimerae and their offspring will provide valuable animal models to study developmental, congenital and biological anomalies, including mental and neurological impairment, due to chromosome abnormalities or mosaicisms in human. In this context, we are now constructing chimaeric mice retaining a hChr.21 to investigate various aspects of Down's syndrome.

Functional expression of a human chromosome in chimaeric mice. That chromosomes from differentiated somatic cells could fulfill their intrinsic function reversibly in a variety of tissues through mouse ontogeny was shown by observed xenogenetic expression of human genes on the transferred chromosomes. We have shown that several human genes on chromosomes transferred from fibroblasts, irrespective of their expression in MH(ES) cells, were expressed in various chimaeric tissues under proper tissue-specific regulation. This phenomenon may result from interaction between *cis*-elements on human sequences and murine *trans*-acting factors, suggesting that these chimerae may be very useful for surveying the compatibility in the regulation of gene expression between human and mouse.

Using a chromosome as a vector, we have introduced very large DNA fragments which exceed the limitation in length of DNA that can be introduced by conventional methods; native expression patterns of introduced genes are reproduced by inclusion of *cis* regulatory elements. By striking example, we have demonstrated the introduction and the expression of human Ig genes in the chimerae. The large number of V segments confers combinatorial diversity in human and mouse Ig genes. In contrast to restricted and abnormal usage of V segments in previously reported human Ig transgenic mice, due to introduction of the constructs lacking most human Ig V segments<sup>27,32,33</sup>, our chimerae represent a repertoire of human Igs very similar to that observed in humans. For example, ten different VH segments scattered throughout

DNA of about 1 Mb<sup>34</sup> have been identified in hμ transcripts of a chimera (Table 2). Interestingly, the V segments most frequently used in human<sup>26</sup> were also commonly detected in the chimerae, suggesting the existence of common preference in recombination or clonal selection of B cells between human and mouse. Additionally, we demonstrated reconstitution of a diverse repertoire of hλ in chimaeric mice and observed specific immune responses to injected human protein. It should be noted that our procedure may provide an alternative approach for generating transgenic mice expressing human Igs; human antibodies with lower immunogenicity<sup>27,32,33</sup>.

Trans-chromosomal mice. The most intriguing question of this work was whether these chromosome 'vectors' could be transmitted to the next generation through the germline, a crucial issue for regarding the potential utility of our procedure. It was thought that many problems remained to be solved for successful transmission of transferred foreign chromosomes<sup>28,35,36</sup>. Indeed, the possibility that an extra human chromosome may inhibit the differentiation of MH(ES) cells into functional germ cells is suggested by observed sterility in some chimerae containing the hChr.14-derivatives. However, on using female chimerae derived from MH(ES) cells containing an hChr.2-derived fragment, we observed efficient transmission of the fragment through the female germline (Table 3). Transmission via the male germline might be more surprising because it has been well recognized that the presence of unpaired chromosomes disrupts the meiotic process in males<sup>35</sup>. However, more detailed analysis of meiotic behavior will be required to elucidate the effect of the presence of the human chromosome fragment on male and female meiosis. Our data also suggests that no structural or functional change of the fragment had occurred during transmission. Although the size and sequence restrictions on efficient germline transmission remains obscure at present, we can now address the issue by using various chromosomes or fragments<sup>37</sup>, including artificially constructed minichromosomes<sup>36,38,39</sup>. Our demonstration provides the first examples of 'trans-chromosomal mice' containing a heritable foreign chromosome and reveals that the chromosome itself can be used as a vector for transgenesis, thereby extending the size limit of DNA fragments currently used to create transgenic mice<sup>2,3</sup>.

Recent technical advances in manipulating mammalian chromosomes have facilitated the introduction of desired mutations into human chromosomes<sup>40</sup> and the construction of human-derived minichromosomes<sup>36,38,39</sup>. Moreover, using the *Cre-loxP* system<sup>4,5</sup>, replacing a specific mouse chromosomal region with the corresponding human chromosomal fragment, will be possible in MH(ES) cells. Such advances, taken together with the technology described here, will permit us not only to elucidate functions of complex genes such as those encoding Igs and T-cell receptors and those responsible for human polygenic diseases, but also to analyse long-range effects such as X-inactivation and genomic imprinting in mice.

## Methods

Microcell-mediated chromosome transfer. Mouse embryonic stem cells (ET2, cultured as described in ref. 15; Lifetech Oriental) were harvested with trypsin, dispersed in ES medium with 18% FBS, and then washed twice with serum-free DMEM. Recipient ES cells (1x10<sup>7</sup> cells) were pelleted by centrifugation and overlaid with microcells or gamma-irradiated microcells<sup>17</sup> purified from 1x10<sup>6</sup> cells of donor A9 clones<sup>41</sup> in serum-free DMEM, followed by re-centrifugation. The pelleted ES cells and microcell mixture was resuspended in 0.5 ml of PEG (1:1.4) solution which was made by dissolving 5 g of PEG1000 (Wako) in 7 ml of serum-free DMEM containing 14% (v/v) of DMSO. After 1.5 min at room temperature, 10 ml of serum-free DMEM was added and centrifuged. The pellet was then resuspended in ES medium and plated onto three 10-cm dishes contain-

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ing feeder layers (G418-resistant primary embryonic fibroblasts, Lifetech Oriental). G418 (300 µg/ml for A9/2-W23 and A9/14-C11) or puromycin (0.75 µg/ml for A9/22-G2) selection was performed 24 h after microcell fusion. The resulting drug-resistant microcell hybrids were cloned between day 7 and day 9 after selection. Each clone was analysed by PCR using IGKC, IGHMC or IGLC primers (see Genomic DNA analysis). Fusion between microcells prepared from A9/2-W23 and TT2 resulted in six G418-resistant colonies. Four of the six clones retained the IGKC marker. Eleven G418-resistant colonies and four puromycin-resistant colonies were also isolated from the fusion experiments using microcells prepared from A9/14-C11 and A9/22-G2, respectively. All clones from A9/14-C11 retained the IGHMC marker and three of the four clones from A9/22-G2 retained the IGLC marker. Gamma-irradiated (30 Gy) microcells<sup>17</sup> from A9/14-C11 were also used and yielded four G418-resistant clones retaining the IGHMC marker. Representative microcell hybrids, MH(ES)2-1 from A9/2-W23, MH(ES)22-1 from A9/22-G2, MH(ES)14-4 and MH(ES)14-5 from A9/14-C11, and MH(ES)14-6y and MH(ES)14-7y from gamma-ray irradiated A9/14-C11 were further analysed and used to produce chimaeras (Table 1b). To introduce the hCF(2-W23) into XO-type ES cells (TT2-F<sup>14</sup>, isolated from TT2; Lifetech Oriental), A9/2-W23P was used as a microcell donor. One of the resultant puromycin-resistant MH(ES) clones, MH(ES)2-21, retained hCF(2-W23) and was used to produce chimaeras (Table 1b).

**Genomic DNA analysis by PCR.** Genomic DNA samples were subjected to PCR using AmpliTaq DNA polymerase (Perkin Elmer). After incubation at 95 °C for 5 min, samples were amplified for 35 cycles: 15 s at 94 °C, 15 s at 55 °C and 20 s at 72 °C (Gene Amp PCR system 9600, Perkin Elmer). Samples were electrophoresed on 2–4% agarose gels and stained with ethidium bromide. Primer pairs for hChr.2 were TPO, D2S171, D2S134, CD8A, D2S438, D2S1331, D2S388, D2S113, D2S373, IL1A, D2S114, D2S156, D2S159 (Research Genetics) and IGKC (Ig κ constant), 5'-TGGAAGGTGGATAACGCCCT-3' and 5'-TCATTCTCCTCCAA-CATTAGCA-3'; IGKV1 (Ig κ variable family-1; L18, ref. 42), 5'-AGTCAGGGCATTAGCAGTGC-3' and 5'-GCTGCTGATGGTGA-GAGTGA-3'; FABP1 (liver fatty acid binding protein), 5'-GCAATCG-GTCTGCCGGAAGA-3' and 5'-TTGGATCATTCTGGAGCCAG-3'. Primer pairs for hChr.14 were TCRA, D14S75, D14S66, D14S43, PCL, D14S78 (Research Genetics) and NP (nucleoside phosphorylase), 5'-ATA-GAGGGTACCCCATCTGG-3' from exon 3 and 5'-AACCAGGTAG-GTTGATATGG-3' from exon 4; MYH6 (α cardiac muscle myosin heavy chain; this primer pair has not been tested for cross reaction to MYH7 gene), 5'-TGTGAAGGAGGACAGGTGT-3' from exon 2 and 5'-TGTAGGGGTTGACAGTGACA-3' from exon 3; IGHMC (Ig μ heavy chain constant exon 4), 5'-GCATCCTGACCGTGTCCGAA-3' and 5'-GGGTGAGTACGAGGTGCCAG-3'; IGHV3 (Ig heavy variable fami-ly-3; VH3-74, ref. 34), 5'-AGTGAGATAAGCAGTGGATG-3' and 5'-CTTGCTACTCCATCACT-3'. Primer pairs for hChr.22 were IGLC (Ig λ constant region), 5'-GGAGACCACCAACCTGCCAA-3' and 5'-GAGAGTTGCGAAGGGGTGACT-3'; MB (myoglobin, exon 3), 5'-TCCAGCTTCTGCAAGAGCAAG-3' and 5'-TGATGTTGGAGGCCAT-GTCC-3'; PVAB (parvalbumin, exon 5), 5'-TGGTGGCTGAAAGCTAA-GAA-3' and 5'-CCAGAAGAATGGTGTCTATTA-3'; IL2RB (IL-2 receptor β chain), 5'-TAGAAGTGTCTGGAGAGGGAC-3' and 5'-CAGCTG-GTACGCGAGTGGTT-3'; ARSA (arylsulfatase), 5'-GGCTATGGGGAG-CTGGGCTG-3' and 5'-CAGAGACACAGGCAGTGAAG-3'; DIA1 (cytochrome b5 reductase, exon 9), 5'-CCCCACCATGATCCAGTAC-3' and 5'-GCCCTCAGAACGACGAAGCAG-3'. Human-specific primer sequences were designed for minimizing cross reaction with murine counterparts using the 'Entrez' sequence database (National Center for Biotechnology Information). Mapping information for markers on the human chromosome was obtained from The Genome Database (Johns Hopkins University School of Medicine).

**Southern blotting.** Digested DNA samples were electrophoresed through a 0.8% agarose gel and transferred to a Hybond N+ membrane (Amersham) in alkaline solution. The membrane was hybridized overnight at 65 °C with <sup>32</sup>P-labelled human L1 repeat probe (1.4 kb EcoRI-BamHI fragment purified from pKA19A plasmid<sup>44</sup>) or human Iκ probe (1.8 kb SacI-SacI fragment<sup>44</sup>) and washed twice at 65 °C in 0.1xSSC, 0.1% SDS for 30 min<sup>45</sup>. Radioactivity on the membrane was visualized by Image Analyzer BAS2000 (Fuji Photo Film).

**FISH analysis and karyotyping.** Preparation of chromosome samples and FISH analysis were carried out by standard methods as described<sup>46</sup>. For digital image microscopy, the Cytovision Probe system (Applied Imaging) was used. For COT-1 painting, digoxigenin (Boehringer) labelled human COT-1 DNA (BRL) was used as a probe, and human-derived chromo-somes were detected with anti-digoxigenin-rhodamine (Boehringer) in mouse chromosomes stained with DAPI (Sigma). The probe for human Iκ was prepared by biotinylating an equimolar mixture of human Iκ (see above) and Cκ (2.5 kb EcoRI-EcoRI fragment<sup>44</sup>) genomic fragments. For two-colour FISH analysis, the Iκ probe and the digoxigenin labelled COT-1 probe were used, and the hybridized probes detected with FITC-avidin (Vector) and anti-digoxigenin-rhodamine, respectively. One round of amplification with biotinylated anti-avidin (Vector) and a second detec-tion with FITC-avidin was performed in order to visualize the signal of Iκ probe. Normal human metaphase spreads were prepared from peripheral blood lymphocytes for *Alu*-PCR painting. Generation of the *Alu*-PCR probe<sup>14,47</sup> using CL1 and CL2 primers and the detection of the signal were essentially performed as described in ref. 13. Signals were visualized by amplification with Texas red labeled Anti-sheep IgG (H+L) (Vector) on human chromosomes stained with DAPI. Fibroblasts from tail of 4–6 week old mice were cultured in DMEM containing 10% FCS and then used for metaphase chromosome preparation.

**Chimaera production.** Ten to twenty MH(ES) cells from each cell line were injected into an 8-cell stage embryo derived from JclMCH(ICR) mice (Clea Japan, Inc.). Injected embryos were then transplanted to the uteri or oviducts of pseudopregnant recipients and allowed to proceed to term. Chimaerism in resulting offspring was determined by the extent of coat pigmentation. The TT2 or TT2F line, derived from C57BL/6xCBA-F1 embryo, gives an agouti coat colour in an albino MCH(ICR) background.

**GPI-assay and dot blotting.** Each tissue sample from 10–16 week old chi-maeras was minced and divided into two aliquots. One was used for the GPI-assay and the other for preparation of genomic DNA. The GPI-assay was carried out as described previously<sup>47</sup>. As MH(ES) clones derived from TT2 were homozygous for the GPI-1B allele, the host embryos used in these experiments were obtained from individuals homozygous for the GPI-1A allele selected from random bred MCH(ICR) stocks. Genomic DNA (1 µg) from each tissue was dotted on a Hybond-N+ membrane using BIO-DOT blotting apparatus (BioRad). The membrane was first hybridized with the murine Thy-1 probe (0.8 kb, *Pst* fragment from pMT8 plasmid, ref. 48) to normalize applied DNA, and then re-hybridized with the human L1 probe. Radioactivity of each dot on the membrane was measured by an image analyzer BAS 2000. To determine the proportion of the cells containing a human chromosome in each tissue, radioactive signal obtained with the L1 probe normalized with the murine Thy-1 probe was interpolated into a standard curve generated by mixing DNA from each MH(ES) clone with various amounts of DNA from L1-negative TT2. More than 90% of metaphase spreads prepared from each MH(ES) cell line contained the transferred human chromosome (determined by FISH, data not shown).

**RNA analysis by RT-PCR.** First-strand cDNA was synthesized with Superscript RTase (BRL) using random hexamers from 2 µg of total RNA extracted from MH(ES) clones and chimaeric tissues. cDNA was amplified by PCR using specific annealing temperatures for each primer pair. PCR products were electrophoresed on 2–4% agar gels and stained with ethid-ium bromide. Integrity of RNA was controlled by the amplification of cDNA generated by the murine β-actin gene (Control Amplimer Sets, Clontech). Based on the sequence data taken from the 'Entrez' sequence database, human-specific primer sequences were designed to enable dis-crimination between products obtained from cDNA and genomic DNA and to minimize cross reaction with murine counterparts, annealing tem-peratures were selected accordingly. Although the primer pairs for FABP1 and IL2RB were based on cDNA sequences, they generated PCR products of a different size to that obtained on PCR amplification of genomic DNA (data not shown).

Primer pairs for chimaera C2-8 were Iκ (61 °C), Cκ 5'-CCAAGCTTCAGAGGAGTCCAGATTTC-3' from Cκ exon and Vκ1BACK<sup>48</sup>, FABP1 (61 °C), 5'-GCAATCGGTCTGCCGGAAGA-3' and 5'-CTTGCTGATTCTCTTGAAGA-3'. Primer pairs for chimaera C14-15 were NP (61 °C), see genomic DNA analysis; TCRA (59 °C), 5'-AAGTTC-



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## Modulation of the Activity of an Avian Gene Transferred into a Mammalian Cell by Cell Fusion

(gene transfer/enzyme induction/hypoxanthine phosphoribosyltransferase/mouse-chick hybrids)

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**ABSTRACT** Mouse A9 cells, deficient in hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), were fused with normal chick erythrocytes and selected in hypoxanthine-aminopterin-thymidine medium for cells with hypoxanthine phosphoribosyltransferase activity. Recovered hybrid cells produced the chick hypoxanthine phosphoribosyltransferase exclusively, as demonstrated by electrophoretic mobility and immunoprecipitation tests, even though no chick chromosomes or chick cell-surface antigens could be identified in the hybrids. Surprisingly, the expression of the chick hypoxanthine phosphoribosyltransferase activity in the mouse/chick hybrids required the presence of aminopterin in the growth medium; in its absence, enzyme synthesis decreased markedly. Because of the rapid and reversible modulation of hypoxanthine phosphoribosyltransferase activity, the hybrid cells could proliferate equally well in media containing hypoxanthine-aminopterin-thymidine or 8-azaguanine. Cellular selection was definitely ruled out as a possible cause. These results confirm previous reports that specific genetic information can be selectively transferred from one cell to another of a distant species. Furthermore, they demonstrate that an avian gene, whose activity is normally expressed constitutively, can become facultative when integrated into a mammalian cell. This seems to be the first instance where heterologous gene activity has been shown to be reversibly modulated in hybrid cells.

Hypoxanthine phosphoribosyltransferase (HPRT), with the electrophoretic characteristics of the normal chicken allozyme, was found in cell hybrids produced by fusion of HPRT-deficient mouse A9 cells with normal nucleated chicken erythrocytes (1). Such hybrids are selected in a medium containing aminopterin, which allows survival only of cells that have regained the ability to produce HPRT. These cells are unusual among cell hybrids in that only the mouse and none of the chicken chromosomes can be identified in them despite their apparent acquisition of the gene for the chicken enzyme. These hybrids also do not express any chick cell-surface antigens. Similar results were obtained when A9 cells were fused with frog erythrocytes (2), or when HPRT-deficient Chinese hamster cells were fused with chick erythrocytes (3). The phenomenon is therefore not restricted to situations in which the recipient cell is of the A9 strain, or the chick nucleus is the donor. It has been postulated that as a result of chromosome fragmentation only a small cytologically unidentifiable seg-

ment of the chicken or frog genome has been incorporated by the recipient cells (1, 3).

The results of the experiments described here confirm these earlier observations, and show further that the transfer of a very small amount of heterologous genetic material is fairly frequent in appropriate fusions, and that this newly acquired genetic characteristic shows considerable stability in many of the recipient cells. Of greater interest is the finding that the expression of the transferred chick *hprt* locus in these hybrids can be modulated rapidly and reversibly as a function of the growth media.

### MATERIALS AND METHODS

**Fusion Procedure.** Mouse heteroploid cells of strain A9 (4), were fused with erythrocytes recovered from 15- or 16-day-old chick embryos in a ratio of 1:5. Before fusion, a subclone of A9 was isolated, grown in medium with 10  $\mu$ g/ml of 8-azaguanine, and tested repeatedly for mycoplasma contamination. This subclone had only barely detectable HPRT activity. Cell fusion was carried out at 4° for 20 min, in the presence of 10<sup>4</sup> HAU of  $\beta$ -propiolactone-inactivated Sendai virus per 10<sup>6</sup> A9 cells, suspended in serum-free McCoy's 5a medium at pH 7.5 (5). After fusion the equivalent of 10<sup>6</sup> A9 cells were distributed in T-75 Falcon plastic flasks in 5a medium supplemented with 20% fetal-calf serum, pH 7.2. After 1-5 days, this was changed to selective (HAT) medium (6) with aminopterin at an end concentration of 4  $\times$  10<sup>-7</sup> M. The flasks contained 10  $\times$  25-mm coverslips for cell sampling or for removal of single colonies.

**Isolation of Hybrids.** Hybrid colonies generally appeared within 3-4 weeks after fusion. Flasks in which a colony grew on a coverslip, or in which only very few colonies developed, were selected for colony isolation. All such primary isolates, although probably derived from single cells, will always be referred to as "colonies."

**Assay of HPRT Activity.** The preparation of cell lysates and determination of HPRT activity according to the DEAE-paper chromatography technique have been described (7). The reaction mixture contained, in a total volume of 50  $\mu$ l: 55 mM Tris-HCl buffer, pH 7.4; 5 mM MgSO<sub>4</sub>; 0.55 mM 5'-phosphoribosyl-1'-pyrophosphate (Calbiochem); 0.50 mM [8-<sup>14</sup>C] hypoxanthine (Schwarz/Mann, 2  $\mu$ Ci/ml); and 10  $\mu$ l of test lysate (1 to 3 mg of soluble protein per ml). All assay were done in duplicate.

**Electrophoresis of Enzymes.** Enzyme electrophoresis was performed on cellulose acetate gels, according to Rattay

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); *hprt*, the genetic locus coding for HPRT; G6PD, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); PGK, phosphoglycerate kinase (EC 2.7.2.3)

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*et al.* (8) for glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase (PGK), and by the method of Shin *et al.* (7) for HPRT.

**Characterization of HPRT with Antiserum against Mouse HPRT.** In addition to electrophoresis, the species of origin of the HPRT activity in the A9/chick erythrocyte hybrids was determined by an immunoprecipitation test in which the sample is treated, in the presence of antiserum prepared in sheep against rabbit globulin, with a specific rabbit antiserum against purified mouse HPRT. Details of the test conditions are described elsewhere (9). Under these conditions, the mouse HPRT is precipitated almost quantitatively while the chick HPRT remains soluble, thereby providing an unambiguous method of discrimination between the two allozyme types.

**Determination of Chick-Specific Cell-Surface Antigens** was done according to a mixed immune hemadsorption method (10).

**Cytologic Techniques.** Giemsa-stained coverslips, removed at 1- and 2-day intervals after fusion, were used to evaluate cell fusion frequency. The method for making chromosome preparations and for staining these by a Giemsa-trypsin banding technique were described earlier (11).

## RESULTS

### Frequency of formation of HAT-resistant colonies

The overall frequency of HAT-resistant colony formation averaged  $2.6 \times 10^{-6}$  per A9 cell fused. This figure represents an underestimate since only the initial group of colonies to appear in each flask (usually 1 to 3) was counted to avoid the inclusion of secondary colonies. In the controls, a total of  $7 \times 10^7$  A9 cells were "fused," with and without virus, with cell-free homogenates of chick erythrocyte or whole embryos, as well as with chick allantoic fluid. No colonies developed in HAT medium. This indicates that the intact chick erythrocyte is necessary for the formation of HAT-resistant cells. Thus it is unlikely that a virus or other contaminant of chick embryos is responsible for their formation.

### Characteristics of HAT-resistant colonies

**HPRT Allozyme in Hybrid Cells.** All of the A9/chick hybrids that were maintained in HAT medium have an HPRT allozyme type whose electrophoretic mobility is indistinguishable from that of the control chick fibroblasts (Fig. 1). In addition, the immunoprecipitation tests show that it is clearly different from mouse HPRT (Table 1). None of the colonies tested had the chick allozymes for G6PD or PGK, indicating that if these markers are also linked to HPRT in the chicken, they had not been transferred with it to the A9 cells.

**Cell-Surface Antigens.** None of the cells of the six colonies tested had detectable chick-cell surface antigens, although the chick fibroblast controls gave strongly positive hemadsorption reactions.

**Chromosome Studies.** Altogether 790 metaphases from 13 freshly isolated colonies were analyzed for chicken chromosomes. Of these, six metaphases had, in addition to the A9 mouse chromosomes, some thin, precociously condensed chromosomes similar to those described in hybrid cells by other authors (12, 13). These resembled chick macro- and micro-chromosomes, but positive identification was not possible. About 45 other metaphases had unidentifiable chromosome

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fragments. All hybrid cells had one or more of the characteristic A9 markers and often had additional chromosome markers unique to each clone. No chick chromosomes could be recognized in any of the cells from later passages. Even the banding methods, however, would not allow the recognition of small chick chromosome fragments if they had been integrated into the mouse chromosomes.

**Hybrid Survival in Selective Media.** Of the 111 primary colonies that developed in six independent fusions, 29 colonies were isolated. To test the stability of the presumed chick HPRT, we first subjected the isolated colonies to reverse selection by transferring them from HAT to 8-azaguanine medium. This should have eliminated cells with stable HPRT activity. Contrary to our expectation, the cells of most colonies continued to proliferate actively. Furthermore, these colonies could be transferred back to HAT medium without apparent decrease in viability, and such serial changes of selective media could be repeated several times. Some of the 29 colonies did behave as expected, however. When two colonies were transferred from HAT to 8-azaguanine medium, extensive cell

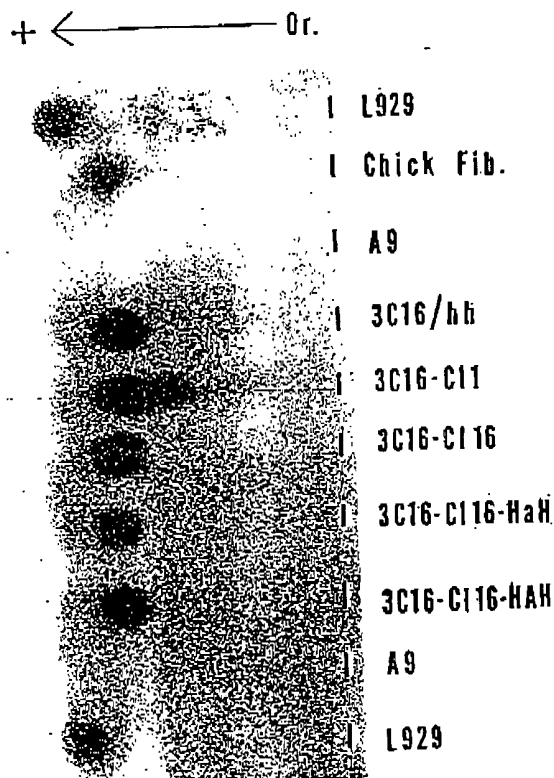


FIG. 1. Electrophoresis of HPRT. Cell lysates were run on cellulose acetate gel for 3 hr in 0.02 M phosphate buffer, pH 7.0. The enzyme activity was revealed by use of [ $^{14}$ C]hypoxanthine on DEAE-paper, followed by autoradiography, according to Shin *et al.* (7). The mouse allozyme (L929) migrates faster than that of chicken (Chick Fib., primary chicken fibroblasts from 16-day-old embryo). The mouse-cell mutant A9 has no detectable HPRT activity in this assay. An original hybrid colony (3C16/hh) and its clonal derivatives, both before and after the disappearance and reappearance of HPRT activity in alternate selective media, all have an HPRT activity of the chick type. Individual hybrid clones used here are identical to those given in Table 1.

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TABLE 1. Characterization of HPRT in A9/chick hybrids

Source of HPRT	Specific activity of HPRT*	Electrophoretic mobility†	Precipitation with antiserum against HPRT			
			HPRT activity remaining (cpm)‡		% Pptd. with antiserum	Species of origin§
			+ Control serum	+ Antiserum against HPRT		
Controls¶						
Mouse HPRT*	25,087	M	3838	100	97.4	M
L929	129	M	1501	75	95.0	M
AR-3 <sup>b</sup>	98	M	628	64	90.2	M
Chick fibroblasts <sup>c</sup>	163	C	606	761	0	C
Chick erythrocytes <sup>d</sup>	48	C	779	807	0	C
Test hybrids¶						
3C14/3-2*	65	C	2144	2182	0	C
3C14/5-19*	74	C	1044	1007	3.5	C
3C14/5-19*	173	C	1122	1152	0	C
3C16/hh*	129	C	445	440	1.1	C
3C16-C1 1-HAH <sup>e</sup>	140	C	1226	1229	0	C
3C16-C1 16*	206	C	1014	1020	0	C
3C16-C1 16-HAH <sup>e</sup>	224	C	674	673	0	C

\* Expressed as nmol of IMP formed per mg of protein per hr at 37°.

† Results from electrophoresis on cellulose acetate gel as in Fig. 1. (M) mouse; (C) chicken.

‡ Results of duplicate assays. The immunoprecipitation mixture contained, in 60  $\mu$ l of total volume: 10  $\mu$ l of test lysate, 1.0  $\mu$ l of rabbit control serum or antiserum against mouse HPRT, and 10  $\mu$ l of sheep antiserum against rabbit IgG. After overnight incubation at 4°, the precipitate was removed by cold centrifugation and the HPRT remaining in solution was assayed with [<sup>14</sup>C]hypoxanthine. Further details in ref. 9.

§ The presumed species of origin of HPRT in the test lysate, as determined by the antigenic crossreactivity to the antiserum against mouse HPRT.

¶ Source of HPRT: \* Purified HPRT from mouse tissues (Shin, unpublished data), used to raise the antiserum against HPRT in rabbit; <sup>b</sup> a revertant of A9 (19); <sup>c</sup> primary chick-embryo fibroblasts in culture; <sup>d</sup> fresh hemolysate of chick erythrocytes from 15-day-old embryos; <sup>e</sup> primary hybrid colonies independently isolated from three different fusions; <sup>f</sup> clonal isolate, after repression and reinduction of HPRT activity by serial growth in HAT-8-azaguanine-HAT media; <sup>g</sup> original clone 16 after growth in HAT medium; <sup>h</sup> clone 16, after serial growth in HAT-5a-HAT media; and <sup>i</sup> same clone 16 as in <sup>h</sup>, but after growth in HAT-8-azaguanine-HAT media (see text and Table 4).

death was observed, but a number of resistant foci developed and these cells were then also able to grow in HAT. In three other colonies all cells died after 2-4 weeks in 8-azaguanine medium. Finally, three colonies from HAT produced survivors in 8-azaguanine medium, but these could then no longer grow in HAT. It is probable that in this last group of hybrids the loss of the HPRT activity was an irreversible process.

**Cloning Experiments.** Cloning experiments were carried out to determine whether genetic segregation and cellular selection

TABLE 2. Plating efficiency of hybrid colony 3C16

Preselection media	Media plated in	Total no. of cells plated	Total no. of clones developed	Efficiency of plating (%)
HAT	HAT	200	152	76
HAT	SAG	200	168	84
HAT	5a	200	161	81
8AG	8AG	200	179	90
8AG	HAT	200	145	73
8AG	5a	200	176	88
5a	5a	200	181	91
5a	HAT	200	165	83
5a	SAG	200	152	76

SAG, 8-azaguanine.

were responsible for the apparent ability of some hybrid cells to survive through serial selection in HAT and 8-azaguanine media, or whether the chick HPRT was being reversibly modulated in all cells. One of the original colonies, 3C16, which had been maintained in HAT for over 100 cell generations, was divided into replicate plates and kept in McCoy's 5a, HAT, or 8-azaguanine medium for 30-60 days. Cell growth was comparable in all three media. Cells from each were then tested for plating efficiency in each of the three media. It is clear from the results in Table 2 that cellular selection does not occur in any of the test media, since regardless of the type of medium in which the cells had been pre-selected before cloning, the plating efficiency is uniformly high. It was found further that clones isolated in a particular medium could grow again in the other two media as well as in media supplemented with thioguanine, guanine, or hypoxanthine and thymidine. However, clones first isolated in 8-azaguanine medium and then maintained for long periods with 8-azaguanine frequently could not be transferred back to HAT medium, even though clones first isolated for a short time in 8-azaguanine medium but subsequently maintained in HAT or normal medium, retained the ability to grow in all other media indefinitely.

**Induction and repression of chick HPRT in hybrid clones**

To rule out the possibility that the A9/chick hybrids might have developed a uniform, specific transport block to 8-azaguanine or thioguanine, thus enabling them to escape the

TABLE 3. Specific activity of HPRT in sample clones and their ability to grow in alternative selection media

Clone no.	Medium in which clone was isolated	Medium in which clone was expanded	Specific activity of HPRT*	Subsequent ability to grow in†				
				HAT	8AG (10 µg/ml)	TG (5 µg/ml)	Guanine (10 µg/ml)	5a
1	8AG	HAT	129	+	+	+	+	+
4	HAT	HAT	140	+	+	+	+	+
5	8AG	8AG	2	-	+	+	+	+
10	8AG	8AG	15	-	+	+	n.t.†	+
13	5a	5a	0	-	+	+	n.t.	+
16	5a	5a	6	+	+	+	n.t.	+

All clones were derived from hybrid colony 3C16, which was itself selected and maintained in HAT medium until it was cloned. 8AG, 8-azaguanine; TG, thioguanine.

\* From cells grown up in the expansion medium, expressed as nmol of IMP formed per mg of protein per hr at 37°.

† (+) cell growth; (-) cell death.

‡ n.t., not tested.

selection against the HPRT-positive cells, we tested 19 primary colonies and 23 independently derived clones at intervals for HPRT activity in the various test media. Without exception, the cells grown in HAT medium always had high HPRT activities, whereas in the other two media it was always low (Table 3). It seems unlikely, therefore, that these products of independent fusion events had all developed transport blocks for both 8-azaguanine and thioguanine.

The results of a more detailed analysis, in which a number of sister clones were carried through serial transfers of selective media in parallel, can be summarized as follows (Table 4): (1) As expected, all cells grown in 8-azaguanine medium had little or no HPRT activity, whereas cells from HAT medium contained amounts of enzyme within the normal range. Most unexpected, however, was the finding that cells maintained in normal, hypoxanthine + thymidine, or guanine medium also showed very little HPRT activity. Indeed, it appears that the hybrid cells express the normal levels of HPRT activity only in the presence of aminopterin. (2) Both the reappearance of activity when the cells are transferred from non-HAT to HAT medium (for instance, in clone 16), and its disappearance when going in the reverse direction (in clone 1), are completely reversible and relatively rapid, requiring less than 5 days. This again suggests that modulation of gene activity, and not cellu-

lar selection or gene segregation, is involved. (3) As shown in clones 10 and 13, prolonged growth in media other than HAT can result in the loss of inducibility, presumably due to a permanent loss of the chick genetic material from the mouse cell. (4) The data shown in Table 1 indicate that the HPRT, which is reversibly and rapidly induced in the hybrid clones, is coded for by the chick *hpri* locus. In hybrid clones 1 and 16, for example, the HPRT activity before and after "reinduction" remains unaltered as the chick type (Fig. 1).

#### Lack of modulation of HPRT activity in chicken cells

The possibility that HPRT activity may be a uniquely inducible function in chicken cells has been ruled out by the following experiments. Primary chick embryonic cells, derived from 15-day-old embryos, were grown in 5a, HAT, hypoxanthine + thymidine, 8-azaguanine (10 µg/ml), or thioguanine (5 µg/ml) medium and assayed at intervals for HPRT. The specific activity of HPRT in the cells remained within normal levels in all the media for up to 12 days (213 ± 35 nmol of IMP formed per mg of protein per hr; range: 125-299). After this, the cells in 8-azaguanine or thioguanine medium began to die. Therefore, the expression of the chick *hpri* locus in these cells appears to be constitutive under the same conditions where it was easily modulated in the A9/chick hybrids.

TABLE 4. Modulation of HPRT activity in hybrid clones: effect of growth medium and duration of culture

Clone no.*	Medium cloned in*	Medium grown in*	Specific activity (S.A.)† of HPRT after specified days in:											
			HAT		HT		8AG		TG		Guanine		5a	
			Days	S.A.	Days	S.A.	Days	S.A.	Days	S.A.	Days	S.A.	Days	S.A.
1	8AG	HAT	5	129	n.t.‡		5	36	4	14	4	27	20	27
							7	1	12	0	11	5		
4	HAT	HAT	6	167	6	35	6	18	5	18	n.t.		6	19
					14	39	15	11					14	20
10	8AG	8AG	No growth		4	27	31	15	4	18	4	34	4	2
13	5a	5a	No growth		n.t.		7	0	6	0	n.t.		39	0
16	5a	5a	6	190	n.t.		5	5	n.t.		n.t.		24	6
			7	206			7	10					31	4
			14	240			14	9						

\* Clone nos. and days in cloning and expansion media correspond to those in Table 3.

† n.t., not tested.

‡ Expressed as nmol of IMP formed per mg of protein per hr at 37°. 8AG, 8-azaguanine; HT, hypoxanthine + thymidine; TG, thioguanine.

## DISCUSSION

These results indicate that the chick *hprt* locus can be selectively incorporated by the mouse cell, and that under certain conditions the normally constitutive expression of this locus may become an inducible function in the recipient mouse cell. To our knowledge, this is the first instance where such a change in the regulation of gene activity has been established in cell hybrids.

Riccardi and Littlefield (14) found that cells from one of four subjects with the Lesch-Nyhan syndrome could survive in either HAT or thioguanine medium. In HAT the cells doubled their HPRT activity, but this was still only about 6% of the activity of normal cells. When aminopterin treatment was stopped, the minimal [<sup>3</sup>H]hypoxanthine uptake of untreated cells was reestablished. Thus, the cells of this patient may also have been subject to modulation, though to a lesser degree than our A9/chick hybrids. Since our work was completed, Davidson and coworkers (15) have reported that the expression of thymidine kinase from inactivated herpes simplex virus introduced into a thymidine kinase-deficient mouse cell could be "suppressed" or "reactivated." In this system, however, the suppression or reactivation event occurred with an extremely low frequency, and could be demonstrated only after a process of cellular selection. It is therefore difficult to compare their results with the rapid and reversible modulation of chick HPRT activity observed in the A9/chick erythrocyte hybrids.

Selective transfer of heterologous genetic material into a mammalian cell has been achieved also with isolated chromosomes (16). When appropriate selection procedures are applied, some of the mouse/human or hamster/human cell hybrids retain only very little human genetic material (17, 18). It should be worthwhile to search for evidence of similar modulatory behavior of selected markers in such hybrids or transformed cells.

The A9-derived hybrid systems offer certain advantages for studies of this kind. The reversion of A9 to the HPRT-positive phenotype has been shown to be extremely rare, maximally  $2.5 \times 10^{-4}$  (19). One of us has provided data that suggest that the HPRT-deficiency in A9 may be due to a point mutation in the structural gene for the enzyme (9). In addition, the product of the *hprt* locus can be analyzed directly, as demonstrated in this study.

In contrast to our results, Bakay *et al.* (20) reported recently that the fusion of chick fibroblasts with mouse 1R cells, a different HPRT-deficient derivative of the mouse L cell (21), resulted in an increased frequency of formation of HAT-resistant colonies. However, the cells recovered from HAT selection contained mouse-type HPRT only. Since there is no independent proof that transfer of chick genetic material has actually taken place at all, the possibility that the HPRT-positive cells may be simple revertants of 1R cannot be ruled out. There are indications, for example, that the fusion process itself may cause a higher frequency of reversion in such mouse-cell mutants (19, 22). Somewhat analogous results have been presented by Boyd and Harris (3), who showed that fusion of thymidine kinase-deficient mouse cells with chick erythrocytes resulted in cells with a mouse-type thymidine kinase. Both observations were interpreted by the authors as evidence that the chick cells used in the fusion had contributed regula-

tor gene(s) to the mouse mutants, whose enzyme deficiencies were *ipso facto* assumed to be due to regulatory mutations. Evidence that these cells have a regulatory mutation is so far unavailable.

The elucidation of the molecular processes responsible for the modulatory behavior observed here may provide new insight into regulatory mechanisms in somatic cells of higher organisms. Aminopterin, the apparent "inducer" in the present system, like other folic acid antagonists, inhibits *de novo* purine synthesis in animal cells by blocking the formyl transfer reactions. It is possible, therefore, that the actual inducing condition is the elevated intracellular concentration of 5'-phosphoribosyl-1'-pyrophosphate, which acts as a substrate for the HPRT reaction, since its cellular concentration should increase in the presence of aminopterin. Another possibility is that the decreased intracellular concentration of IMP derepresses the purine salvage pathway enzyme. If either of these is true, other antimetabolites such as azaserine, which inhibit the purine synthetic pathway by a different mechanism, should also be effective inducing agents. Alternately, aminopterin could interact directly with the inserted chick genetic material. A fourth possibility, that of aminopterin acting as an activator or stabilizer of the HPRT molecule already synthesized, is effectively ruled out by the data provided here.

This work was supported by NIH Genetics Center Grant GM 19100. H.P.K. thanks Prof. Henry Harris, University of Oxford, for hospitality during the initial phases of this work, and Prof. J. F. Watkins for assistance with the hemadsorption method. We thank Miss Tina Ditta and Mr. André Brown for excellent technical assistance. H.P.K. holds an NIH Career Development Award (GM25,260).

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## Original Articles

Cytogenet. Cell Genet. 24: 129-137 (1979)

### Pattern of segregation of chicken HPRT phenotype in Chinese hamster-chick red blood cell hybrids

I. RASKÓ, S.L. PÉTER, K. BURG, L. DALLMANN, and G. BAJSZÁR

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**Abstract.** The pattern of segregation of hypoxanthine phosphoribosyltransferase (HPRT, E.C. 2.4.2.8) was determined in synchronized Chinese hamster-chick red blood cell hybrids. Three hybrid lines were synchronized at the G1-S boundary. Bromodeoxyuridine pulses were subsequently applied throughout the S phase, and the frequency of the segregant clones was determined. It was found that the segregation of the chicken-specific HPRT phenotype associated with the loss of a chromosome was potentiated by bromodeoxyuridine administered during the first hour following release of the block.

The process of DNA replication is not only nonrandom in eukaryotes but has a specific temporal order. In tissue culture cells both DNA-RNA hybridization techniques (BALÁZS et al., 1973; STAMBROOK, 1974) and sequential mutagenetic induction have been applied to determine the temporal order of replication of different markers (KAJIWARA and MUELLER, 1964; SALGANIK, 1972; SUZUKI and OKADA, 1975; AEBERSOLD and BURKI, 1976; LOUGH and BISCHOPP, 1976; RASKÓ et al., 1976; CHANG and BASERGA, 1977).

In hybrid cells, only cytogenetic techniques have been used to detect the replica-

tion pattern of those parental chromosomes which have remained in the cells. In these studies, hybrids between Chinese and Armenian hamster cells (SONNENSCHNEIN, 1970), mouse and Chinese hamster cells (GRAVES, 1972; MARIN and COLETTA, 1974), and mouse and human cells (LIN and DAVIDSON, 1975) were used. These investigations showed that the characteristic time pattern of chromosome replication was preserved and that the timing of chromosome replication was determined independently in each chromosome.

We have shown previously that bromodeoxyuridine (BrdU) treatment enhances the loss of expression of chicken-specific hypoxanthine phosphoribosyltransferase (HPRT, E.C. 2.4.2.8) from Chinese hamster chick red blood cell hybrids (RASKÓ et al., 1978). In this paper we present evidence

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that the effect of BrdU on such segregation is restricted to a defined period of the S phase in hybrid cells and that the loss of HPRT expression is always accompanied by the loss of a presumptive chicken chromosome.

### Materials and methods

**Cell culture and media.** CHO K1 (HPRT<sup>+</sup>) cells (Kao and Puck, 1967) and a 6-thioguanine (TG) resistant mutant of this cell line (designated T4) were grown in F12 medium (GIBCO) supplemented with 5% fetal bovine serum (Flow) (F12+5FC medium).

T4 cells and chick red blood cells obtained from 9-day-old chick embryos were fused with UV-inactivated Sendai virus according to the method of MARCUS and WATKINS (1965). Several colonies were isolated from different plates and maintained in F12+5FC medium supplemented with the HAT components. In this study, three hybrid lines isolated from different dishes were used (T4C12, T4C15, and T4C21).

**Cell synchronization.** The HPRT<sup>+</sup> CHO K1 and the hybrid cells were synchronized in HAT medium at the beginning of S phase by excess thymidine followed by a hydroxyurea block, as described earlier (RASKÓ et al., 1976).

**Analysis of cellular progression throughout the cell cycle.** The mitotic index was determined after the indicated time intervals by accumulating metaphases with 0.2 µg/ml of Colcemide (Fluka), which was added to the cultures after the hydroxyurea block was released. For the determination of <sup>3</sup>H-TdR incorporation, 10<sup>5</sup> cells were synchronized in 60-mm plastic Petri dishes. After synchronization, 0.5 µCi/ml <sup>3</sup>H-TdR (specific activity, 19.6 mCi/mole, UVVVR, Prague) was added. At the indicated times the medium was removed, and the dishes were rinsed twice with 5 ml of saline, twice with 5 ml of cold 5% TCA, and then stored overnight in 5% TCA at 4° C. After removal of the TCA, 0.3 ml 1N NaOH was added for an additional 5 h. Aliquots were transferred to scintillation vials and counted in an LKB liquid scintillation spectrometer using Bray's scintillation fluid. The results were expressed in terms of counts per

minute per microgram of protein. The protein content was determined by using the method of LOWRY et al. (1951).

**Bromodeoxyuridine mutagenesis and selection of HPRT<sup>-</sup> mutants from CHO K1 cells.** The conditions for the induction of mutations and the selection of the HPRT<sup>-</sup> mutants were as described earlier (RASKÓ et al., 1976).

**Bromodeoxyuridine pulses and selection of HPRT<sup>-</sup> clones in hybrid cells.** After the hydroxyurea block, the hybrids were pulse-labeled with 10<sup>-4</sup> M BrdU in TdR minus F12+5FC medium for 1-h periods throughout the S phase and, in the case of T4C15 cells, during the G2 phase as well. After the BrdU pulses, 10<sup>-4</sup> M TdR chases were used. Synchronized cells without BrdU pulses served as controls. After the BrdU was removed, the cells were incubated in F12+5FC medium for 5 days. This period of time, as observed previously (RASKÓ et al., 1978), proved to be optimal for the manifestation of BrdU's effect on segregation. At this time, aliquots of 10<sup>5</sup> cells were plated on 100-mm glass Petri dishes containing F12+5FC medium and 5 µg/ml TG, and the plating efficiency was determined in F12+5FC medium. In the TG-containing back-selection medium, only the HPRT<sup>-</sup> cells are able to form colonies. After 7 days of incubation, several colonies were picked up for further cultivation and the remaining colonies were fixed and stained. The frequency of HPRT<sup>-</sup> colonies was calculated according to the following equation:

Frequency of HPRT<sup>-</sup> colonies =

Number of HPRT<sup>-</sup> colonies  
in TG medium  $\times 100$

Number of cells selected  $\times$  Plating  
efficiency (%)

**HPRT assay.** The HPRT enzyme converts labeled hypoxanthine to labeled inosine monophosphate (IMP) in the presence of phosphoribosylpyrophosphate. The IMP so produced can be specifically bound to Whatman DE 81 paper. The composition of the assay mixture was that published by WAIN et al. (1975). The reaction time in our case was 7 min.

The heat-inactivation experiments were carried out according to the method of SHARP et al. (1973).

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The protocol was as follows: The cells were lysed in Triton X100 containing buffer solution, and the HPRT enzyme was partially purified by ultracentrifugation and by molecular sieving on a Sephadex G200 column. The enzyme was stabilized by bovine serum albumin and concentrated by filtration on an Amicon PM-10 filter. The heat inactivation was carried out at 80° C. The remaining enzyme activity was measured from the supernate of the inactivated enzyme.

**Cytologic techniques.** The chromosomes of 50 metaphases were counted from the T4 parent, the three hybrids, and three segregants. The modal chromosome number was determined according to the method of HAM and PUCK (1162). The diagrams were constructed from Giemsa-stained slides. The G- and C-chromosome banding method are those of WORTON (1972) and SUMNER et al. (1971), respectively.

## Results

The HPRT<sup>+</sup> CHO K1 and the T4-chick hybrid cells were synchronized at the G1-S boundary and the progression of the cells after synchronization was monitored as described in the Materials and methods. Since the accumulation of mitotic cells in the presence of Colcemide was found to be similar in all three hybrids, the kinetics of DNA replication was determined only for the T4C21 hybrid and the CHO K1 cell line (fig. 1). Incorporation of <sup>3</sup>H-TdR leveled off at about 4.5 h, indicating that the duration of the S phase in these cells was 4–5 h.

Bromodeoxyuridine pulses applied at different times during the S and G2 phases had different effects on the induction of the HPRT<sup>+</sup> phenotype. The drug significantly increased the frequency of the HPRT<sup>+</sup> colonies in all three hybrids if it was administered during the first hour of the S phase (table I). The data presented in table I for the BrdU-induced frequency of HPRT<sup>+</sup>

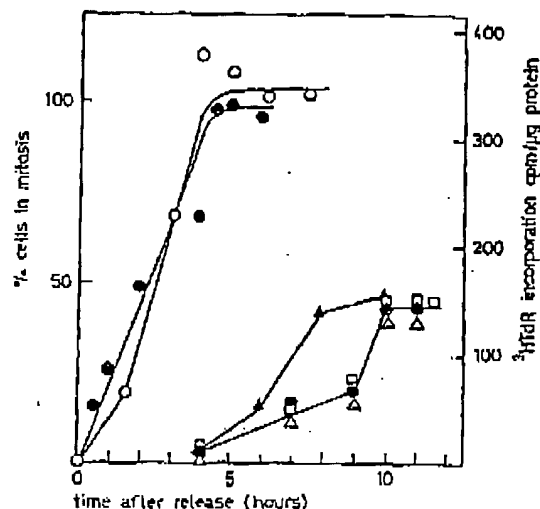


Fig. 1. Cell progression after synchronization. At left, percentage of cells in mitosis: (▲) CHO K1; (●) T4C12; (□) T4C15; (△) T4C21. At right, <sup>3</sup>H-TdR incorporation into synchronized cells: (●) CHO K1; (○) T4C21.

expression in the three hybrids are two to three orders of magnitude higher than the BrdU-induced mutation frequency for the HPRT gene, showing the highest frequency in the second hour in synchronized CHO K1 cells.

The HPRT<sup>+</sup> colonies developed after BrdU treatment were characterized by several means: The hybrids produced the chicken-type HPRT enzyme, as judged by heat inactivation (fig. 2 and table II). The specific activity of the HPRT enzyme in several randomly selected BrdU-induced clones was zero or negligibly low as compared to that of the hybrids (table II).

The BrdU-induced HPRT<sup>+</sup> colonies were not able to grow in HAT medium, even if they were tested at different times after the removal of 6-thioguanine.

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Table I. The effect of BrdU pulses on the induction of the HPRT<sup>-</sup> phenotype in synchron. hybrids and CHO K1 (HPRT<sup>+</sup>) cells

Cells	Time of BrdU administration (h) <sup>1</sup>	Number of cells	Number of TG resistant colonies	Induced frequency of TG resistant colonies per 10 <sup>6</sup> cells <sup>2</sup>
T4C12	Control	2 × 10 <sup>5</sup>	492	—
	0		1202	355
	1		674	91
	2		654	81
	3		706	107
T4C15	Control	2 × 10 <sup>5</sup>	686	—
	0		1158	236
	1		754	34
	2		752	33
	3		760	37
	4		798	56
	5		812	63
	6		692	3
T4C21	Control	3 × 10 <sup>5</sup>	68	—
	0		252	61.3
	1		125	19
	2		76	2.6
	3		78	3.3
CHO K1	Control	6 × 10 <sup>6</sup>	0	—
	0		19	0.27
	1		26	0.32
	2		7	0.11
	3		1	0.01

<sup>1</sup> One-hour BrdU pulses were administered to synchronized cultures; 0 hour should be considered the time of release of the cells from the synchronization blockade.

<sup>2</sup> Induced frequency × 10<sup>-6</sup> = number of TG-resistant colonies × 10<sup>-6</sup> of BrdU-treated sample - number of TG-resistant colonies × 10<sup>-6</sup> of untreated sample.

hybrids was always 21, whereas the parental T4 cells and the HPRT<sup>-</sup> colonies possessed only 20 chromosomes (table II). The extra chromosome in all the hybrids was always a microchromosome, which proved to be too small to be characterized by conventional banding techniques (fig. 3). The num-

ber of cells per hybrid line having this chromosome was also determined (table II). The loss of the microchromosome is characteristic for the HPRT<sup>-</sup> colonies (fig. 4). This chromosome is also missing from those HPRT<sup>-</sup> cells that appeared spontaneously in the hybrid cultures (data not shown).

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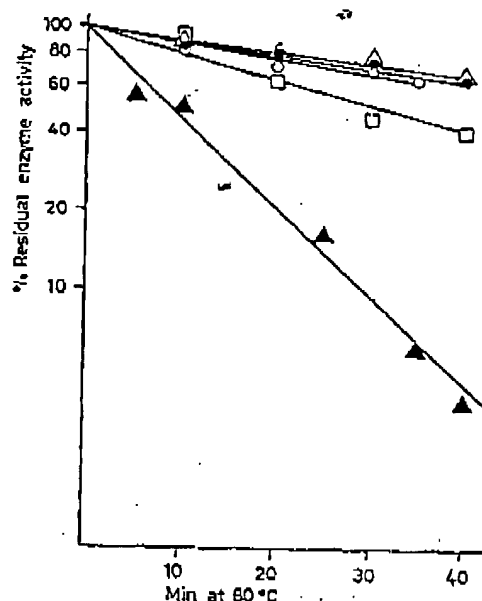


Fig. 2. Residual enzyme activity following heat inactivation of HPRT obtained from the different cell lines studied: (▲) CHIO K1; (○) CRHC; (●) T4C12; (□) T4C15; (△) T4C21.

## Discussion

In the present study, three independently isolated Chinese hamster-chick red blood cell hybrids were used; all produced chicken-type HPRT and contained one presumably chick microchromosome. In the literature there are conflicting reports whether chick chromosomes can be detected after fusion of chick red blood cells with other cell lines. Some of the authors reported the absence of visible chick chromosomes in such hybrids, although chicken-specific gene products were clearly demonstrable (SCHWARTZ et al., 1971; BOYD and FLARKIS, 1973; KLINGER and SHIN, 1974). Recently, however, chicken chromosomes functioning in the biosynthesis of adenine and the chromosome carrying the thymidine kinase gene were identified in hybrid cells (KAO, 1973; LIUNG et al., 1975).

Previously, BrdU was shown to increase



Fig. 3. a, Giemsa-banded chromosomes of T4C21 hybrid, b, C-banded chromosomes of T4C21 hybrid. The chicken-type microchromosome is indicated by the arrow.

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Table II. Properties of parental cells, hybrids, and their segregants

Cell	HPRT		Modal chromosome number	Number of hybrid cells with the microchromosome/50 metaphases	Growth in	
	Heat inactivation <sup>1</sup>	Specific activity <sup>2</sup>			HAT	6-thioguanine
CHO K1 (HPRT <sup>+</sup> )	7.5	365.7	20	-	+	-
T4	-	0	20	-	-	+
CRBC <sup>3</sup>	54.3	5.3	nd <sup>4</sup>	-	-	-
T4C12	58.5	46.39	21	38	+	-
Segr. 1	-	0.1	20	-	-	+
2	-	0	20	-	-	+
T4C15	31.3	56.15	21	36	+	-
Segr. 1	-	0	20	-	-	+
2	-	0	20	-	-	+
T4C21	66.0	52.85	21	41	+	-
Segr. 1	-	0	20	-	-	+
2	-	0.1	20	-	-	+

<sup>1</sup> Heat inactivation:  $t_{1/2}$  (min) given.<sup>2</sup> Specific activity: IMP  $\times 10^{-12}$  mole/ $\mu$ g/h.<sup>3</sup> CRBC = chick red blood cell.<sup>4</sup> nd = not determined.

the segregation of chicken-specific HPRT from Chinese hamster-chick red blood cell hybrids and was found to be effective only in S phase (RASKÓ et al., 1978). The loss of chicken-specific HPRT expression was always accompanied by the loss of a microchromosome originating, probably, from the chicken parental cell.

In the present study we demonstrated that the frequency of BrdU-induced segregation events was on the order of magnitude of  $10^{-5}$ , while the BrdU-induced mutation frequency for the Chinese hamster HPRT gene was  $10^{-6}$  (table I). The possibility that BrdU influences the stability of the presumptive chick microchromosome, rather

than induces mutations of the chicken specific HPRT gene, is supported by the lack of the presumptive chick microchromosome in the HPRT<sup>-</sup> segregants and by the three order of magnitude difference between the frequencies of segregation and mutation.

We have also shown in this study that BrdU-induced segregation of the chick-specific HPRT phenotype can be assigned to a definite period of the S phase of the hybrid cells; BrdU exerted its effect in the first hour of the S phase in all three hybrid lines. Recently, it was suggested that DNA synthesis in mid- or late-S depends upon the synthesis of previously replicating DNA and that this process can be disturbed by the

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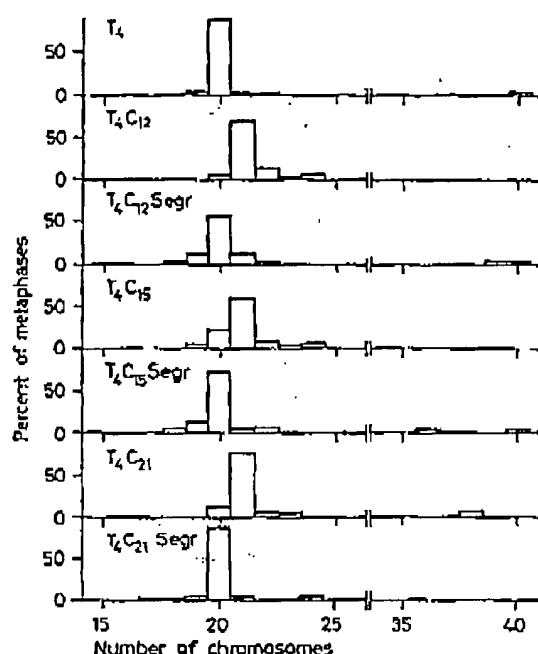


Fig. 4. Distribution of chromosome counts (based on 50 metaphases of each cell type) in the hybrids and their segregants.

incorporation of BrdU into DNA, followed by visible light illumination (HAMILIN, 1978). On the other hand, phosphorylated metabolites of BrdU were shown to inhibit the fusion of myoblasts (ROGERS et al., 1975) and to be responsible for BrdU-induced mutations in Syrian hamster cells (DAVIDSON and KAUFMAN, 1978). At present it is difficult to judge whether the incorporated BrdU or the phosphorylated metabolites of the drug are responsible for the loss of the presumptive chick microchromosome. The fact that our cultures were not protected from room lights during manipulations may favor the importance of BrdU incorporation into DNA, but the role of phosphorylated BrdU metabolites cannot be ruled out.

Several laboratories have demonstrated that BrdU induces mutations in genes that replicate in the presence of BrdU (SALGANIK, 1972; DAVIDSON and BICK, 1973; SUZUKI and OKADA, 1975; AEBERSOLD and BURKI, 1976; RASKÓ et al., 1976; CHANG and BASIRGA, 1977; KAUFMAN and DAVIDSON, 1978). Since the maximum frequency of the HPRT<sup>-</sup> phenotype in CHO cells occurs during the second hour of the S phase (AEBERSOLD and BURKI, 1976, and this study), it may be concluded that the gene(s) responsible for the manifestation of the HPRT<sup>-</sup> phenotype replicate during this period.

In our case, the pattern of BrdU-induced segregation of chicken-specific HPRT phenotype was similar in all three hybrid lines, showing a peak during the first hour of the S phase, and the loss of the HPRT phenotype was always accompanied by the loss of a chromosome in our hybrids. Therefore, the BrdU-induced loss of the microchromosome also seems to be a specific event, and BrdU possibly affects the early replicating DNA involved in stabilizing the microchromosome in the hybrid cells.

In this type of experiment the degree of synchronization is a critical factor. It has been shown previously that CHO K1 cells can be synchronized with excess thymidine, hydroxyurea treatment disturbing neither the normal progression of the cells through the S and G2 phase nor the chromosome replication pattern (RASKÓ et al., 1976). Although the cell cycle seemed to be normal after synchronization in this study, we cannot exclude the possibility that the drugs used for synchronization might have affected the replication pattern of the chicken DNA. The minor effect of BrdU on segregation in the latter part of the S phase can be ac-

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counted for by the partial desynchrony of the cultures, which also shows on the accumulation of the mitotic cells (fig. 1).

In our experiments the loss of a chromosome from a hybrid cell can be easily monitored by the loss of a phenotypic marker, and the frequency of its segregation can be modulated. This and related systems may provide excellent tools for elucidating the factor(s) involved in stabilizing a chromosome in a hybrid cell.

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## Gene Targeting to the Centromeric DNA of a Human Minichromosome

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FRANCO TREDICI, MIRELLA BENSI, and LUIGI DE CARLI

### ABSTRACT

A human supernumerary minichromosome (MC), previously identified as a derivative of chromosome 9, has been introduced into Chinese hamster ovary (CHO) cells by means of cell fusion. A hybrid clone containing the MC as the only free human chromosome was isolated. A selectable marker gene (*neo*) inserted into a yeast artificial chromosome (YAC) has been successfully targeted to the MC centromeric DNA via co-transfection with chromosome-9-specific  $\alpha$  satellite DNA. *In situ* hybridization and Southern blotting experiments demonstrated that the intact *neo* gene was integrated into the MC centromeric DNA. Studies on the clonal distribution and on the stability of the MC either in the presence or in the absence of the selective agent have been carried out. The MC is susceptible to further manipulations and may thus represent a model for the construction of a large-capacity vector for somatic gene therapy.

### OVERVIEW SUMMARY

The bacterial *neo* gene as a selectable marker has been targeted to the centromeric DNA of a human accessory minichromosome (MC) via co-transfection with specific  $\alpha$  satellite DNA sequences. The *in vitro* stability of the MC has been demonstrated. Experiments of size reduction by X-ray exposure and telomere-directed fragmentation are currently made in view of the potential function of the MC as a vector for somatic gene therapy.

### INTRODUCTION

THE METHODS FOR THE INTRODUCTION OF FOREIGN GENES INTO mammalian cells make use of viral vectors, calcium-phosphate-mediated DNA precipitation, lipofection, cell fusion, microinjection, and electroporation. The most efficient method is retroviral-mediated gene transfer, which has been successfully applied to somatic gene therapy in human patients. More recently, yeast artificial chromosomes (YACs) containing large human DNA inserts have been introduced into mammalian cells by microinjection or lipofection (Gnirke *et al.*, 1993).

Because integration via homologous recombination is a rare occurrence in mammalian cells, current experiments of gene transfer are generally affected by random integration of exoge-

nous DNA. This can produce undesired effects due to insertional mutagenesis and uncontrolled expression of the transgene (Watson, 1993). Moreover, viral vectors cannot accommodate long stretches of DNA, such as whole genes with the appropriate transcription signals. Therefore, a suitable vector for gene transfer should be a "large size capacity vector" with minimum interference with the host cell genome (Brown, 1992). Minichromosomes (MC) maintained as stable and heritable supernumerary elements in normal individuals might possess these prerequisites.

In human beings, supernumerary MC are found in about 0.024% of live newborns; 40% of the cases are of familial origin. In most cases these accessory chromosomes have no phenotypic effect, mainly because they contain highly repeated DNA sequences (Buckton *et al.*, 1985). Linear MC, contained in *in vitro* cultured human cells, can possess a high mitotic stability, as demonstrated by the results of our investigations on a lymphoblastoid line carrying a human supernumerary MC, found in a patient with a mosaic 46,XX/47,XX+9/47,XX+mar (Raimondi *et al.*, 1991). The MC was separated from mitotic cells to construct a DNA library to be used for its molecular dissection. The isolation was obtained using two different methods, namely flow sorting and microdissection (Ferretti *et al.*, 1991; Raimondi *et al.*, in preparation). This marker chromosome could thus be identified by reverse *in situ* hybridization as a derivative of the p13-cen region of chromosome 9. We

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have also shown that the MC carries centromeric satellite DNA sequences and normal telomeres. The organization of the centromeric region of the MC was analyzed by two-color fluorescence *in situ* hybridization (FISH) with probes for varying subfamilies of  $\alpha$ -satellite and satellite III DNA (Ascenzi *et al.*, 1995). MC with these characteristics can thus well be used as representative models for functional human chromosomes.

In the present paper, we present the results of co-transfection experiments aimed at introducing a selectable marker gene into the MC centromeric DNA, in view of the possibility of engineering the MC making it usable for studies on its potential vector function in somatic gene therapy. The MC was isolated in a human/hamster somatic hybrid (Raimondi *et al.*, 1995). To confer an adaptive value in culture to the MC-containing hybrid cells, gene targeting experiments were designed to insert the *neo* gene into the MC centromeric DNA via co-transfection with chromosome-9-specific  $\alpha$  satellite DNA. A YAC containing the *neo* gene under the control of SV40 promoter (pN415, Traver *et al.*, 1989) was used for transfection. Attempts at fragmenting the MC are currently being made to reduce it to a minimum-size human chromosome, in view of its possible use as a shuttle vector between mammalian and yeast cells.

Lipofection was chosen as the delivery system because it allows transfer of large DNA fragments (Choi *et al.*, 1993). One of the transformants showing hybridization signals only on the MC centromere, when probed with the *neo* gene and with the chromosome-9-specific  $\alpha$  satellite by two-color FISH, was further analyzed. Southern blot experiments allowed us to demonstrate that the intact YAC had been integrated.

## MATERIALS AND METHODS

### Cell lines

Human HT180 cells were obtained from the American Type Culture Collection; a cell line was originally derived from a human sarcoma (Rasheed *et al.*, 1974). The chromosomal constitution is heteroploid with a modal chromosome number of 71 (range 63–88). The modal number of chromosomes 9 is 3.

The human/hamster hybrid clone was isolated after polyethylene glycol (PEG) fusion of human lymphoblastoid cells carrying the MC with HPRT<sup>+</sup> CHO cells. The hybrids were selected in HAT medium.

To obtain a clone containing the MC as the only free centric human chromosome, serial subcloning was performed. Each step was followed by morphological examination of chromosomes and *in situ* hybridization with MC-specific probes (Raimondi *et al.*, 1995).

### Chromosome preparation, probes, and *in situ* hybridization

For chromosome preparation, 10<sup>5</sup> cells of each hybrid clone were seeded onto a 24 × 24-mm coverslip and incubated overnight in complete medium. For RBG banding, bromodeoxyuridine (BrdU) (30  $\mu$ g/ml) was added to the cultures 7 hr prior to harvesting. After a 2-hr treatment with Colcemid (GIBCO, 0.03  $\mu$ g/ml), the coverslips were processed according to the standard method, based on hypotonic swelling in 0.075 M KCl and fixation in methanol/acetic acid (3:1).

The following probes were used for FISH: pMR9A, identifying a chromosome 9-specific  $\alpha$  satellite DNA subfamily (Rocchi *et al.*, 1991); pSV2neo, containing a bacterial *neo* gene under the control of SV40 promoter (Southern and Berg, 1982); human genomic DNA purified from a peripheral blood sample; genomic DNA from the MC-containing hybrid amplified by *Alu* polymerase chain reaction (PCR) (primers, 5'-TGAGACG-GAGTCTCGCT-3'; buffer, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris HCl pH 9, 0.01% Triton X100, 2% formamide; 30 cycles, denaturation at 94°C 30 sec, annealing at 50°C 45 sec, elongation at 72°C 2 min).

All the probes were labeled using a nick-translation reagent system (GIBCO) and Bio-11-dUTP (Sigma) according to the protocols provided by the suppliers. The labeled probes were resuspended in hybridization buffer (50% formamide, 10% dextran sulfate, 1× Denhart's solution, 0.1% SDS, 40 mM Na<sub>2</sub>HPO<sub>4</sub> pH 6.8, 2× SSC) at a final concentration of 1–5  $\mu$ g/ml and denatured at 70°C for 10 min.

*In situ* hybridization was performed, essentially, as previously described (Pinkel *et al.*, 1986). In brief, slides were treated with RNase (type III, Sigma) at 37°C for 1 hr and dehydrated through an ethanol series before denaturation in 70% formamide/2× SSC. The hybridization was performed overnight at 42°C. Stringent washings were performed in 50% formamide/2× SSC at 39°C.

For probe detection, the slides were incubated first with 5  $\mu$ g/ml fluorescein isothiocyanate (FITC)-conjugated avidin distinct cell sorter grade (DCS) (Vector Laboratories), then with 5  $\mu$ g/ml biotin-conjugated goat anti-avidin D antibody (Vector Laboratories), and finally with FITC-conjugated avidin. Slides were counterstained with propidium iodide (0.5  $\mu$ g/ml) and mounted in Tris-HCl pH 7.5, 90% glycerol containing 2% DABCO antifade (1,4-diazobicyclo-(2.2.2) octane) (Sigma).

Slides were scored under a Zeiss Axioplan fluorescent photomicroscope using a filter-set for blue and green excitation (Zeiss 487909, Blue 450–490 and 487915, green H 546). Because the microscope was not equipped with a camera and an image-processing device, to obtain a better signal-to-noise ratio, some pictures were digitalized using a photo-scanner connected to an image processing program.

### Transfection

In the transfection experiments, cloned circular DNA was used. HT180 and MC-containing hybrid cells were transfected with pMR9A plasmid DNA and with DNA from a YAC containing the *neo* gene under the control of SV40 promoter (pN415, Traver *et al.*, 1989).

A total of 5 × 10<sup>5</sup> cells were plated in 60-mm Petri dishes (NUNC) and incubated for 24 hr in RPMI-1640 serum-free medium. Each dish was treated for 6.5 hr with Transfectam reagent (Promega), containing 25  $\mu$ g of pMR9A and pN415 DNA, respectively, basically following the protocol suggested by the supplier. After 24 hr the selective agent (G418, 500  $\mu$ g/ml, Sigma) was added. G418-resistant clones appeared 9 days after transfection.

### Southern blotting

DNA from pN415 and from the Tr4 clone were digested with *Pvu* I (5 U/ $\mu$ g, 12 hr) and run on a 0.8% agarose gel (40

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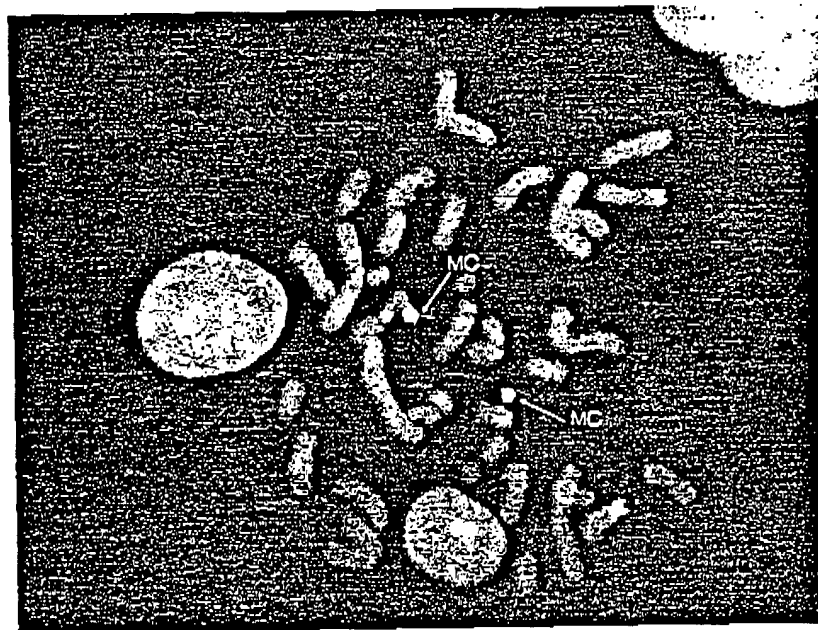
**FIG. 1.** Reverse chromosome painting. The probe used is genomic DNA from the hybrid clone 83/20/4 amplified by *Alu* PCR. Top. FISH analysis of human male lymphocytes. The pericentromeric region of two homologous C-group chromosomes, a distal fragment of the long arm of two homologous C-group chromosomes, and a single C-group chromosome are painted. Bottom. RBG banding allows identification of the labeled chromosomes. The pericentromeric region of both chromosomes 9, the distal region of the long arm of both chromosomes 6 (q22-q27), and the whole X chromosome are painted. It can be concluded that these chromosome fragments were originally present in the hybrid clone.



volt, 24 hr); then they were blotted on a nitrocellulose filter (Amersham). The filter was hybridized with the pSV2neo and with the pMR9A probes labeled with  $^{32}\text{P}$  by random priming using a commercial kit (Megaprime, Amersham). After 24 hr of autoradiographic exposure, the film was developed.

## RESULTS

A hybrid clone (83/20/4) obtained after cell fusion, chromosome segregation, and subcloning was selected for further analyses because it contained a complete CHO chromosome set plus the MC, which was presumably the only residual centric chromosome of human origin. To check this possibility, fluorescence *in situ* hybridization (FISH) experiments were performed on cells from the hybrid clone, using human genomic DNA as a probe. They demonstrated that, in addition to the MC, two chromosome fragments of human origin were translo-



**FIG. 2.** Two-color FISH with the pSV2neo (red label) and the pMR9A (white label) probes on the transformed hybrid clone Tr 4. This mitosis is tetraploid and carries two MCs both showing co-hybridization of the two probes on the same site. A single hybridization site, localized on the MC, can be seen with each of the probes.

cated on Chinese hamster ovary cell (CHO) chromosomes. To identify the translocated human chromosome fragments, DNA was extracted from clone 83/20/4, amplified by *Alu* PCR, and used for reverse chromosome painting on human male lymphocytes. As shown in Fig. 1, the pericentromeric region of both chromosomes 9 is labeled as expected. In addition to these chromosomes, the distal region of the long arm of chromosome 6 (q22-q27) and most of the X chromosome are painted. The presence of the human X chromosome is not surprising because HGPRT<sup>-</sup> CHO parental cells were used for fusion and the hybrid clones were selected in HAT medium (Raimondi *et al.*, 1995).

To target a selectable marker gene to the MC centromeric DNA, co-transfection experiments were performed with the pMR9A sequence and the *neo* gene, which is known to confer dominant G418 resistance to mammalian cells. The *neo* gene was carried by a YAC (pNN415, Traver *et al.*, 1989) and lipofection with the lipid DOGS (Transfectam, Promega) was used as the DNA delivery system. Pilot experiments were carried out on a human cell line (HT1080) at high transfection efficiency to determine the frequency of targeting events to chromosome 9  $\alpha$  satellite sequences. The frequency of G418-resistant clones was  $5 \times 10^{-4}$ ; 75 metaphase spreads from 10 independent resistant clones were scored after FISH with the *neo* gene as a probe. The total number (*N*<sub>t</sub>) of available target sites (AS), including both the centromeric and noncentromeric regions, for all the scored mitoses of each clone, was estimated as follows: the total length (in  $\mu$ m) of human chromosomes of the normal complement (Paris Conference, 1971) was adjusted for HT1080 model chromosome number (*N* = 71). The total length of the HT1080 chromosome complement was divided by the unit length, corresponding to the minimum distance between two clearly distinguishable hybridization signals, which in our experience is equivalent to half the size of the MC, that is 0.5  $\mu$ m. Therefore:

$$N_t AS = 272/0.5 \mu m = 544 \mu m$$

The targeting frequencies (Tf) for the three classes of labeled sites (LS)—non cen, cen 9(MC), cen 9(MC)—have been calculated as a percent of labeled to available target sites (AS), according to the following general formulas:

$$\begin{aligned} Tf_{non\ cen} &= \frac{No\ non\ cen\ LS}{No\ non\ cen\ AS} \times 100 \\ Tf_{cen\ 9\ (MC)} &= \frac{No\ cen\ 9\ (MC)\ LS}{No\ cen\ 9\ (MC)\ AS} \times 100 \\ Tf_{cen\ 9\ (MC)} &= \frac{No\ cen\ 9\ (MC)\ LS}{No\ cen\ 9\ (MC)\ AS} \times 100 \end{aligned}$$

For the HT1080 cell line the median values of the  $Tf_{non\ cen}$ ,  $Tf_{cen\ 9}$ , and  $Tf_{cen\ 9}$  calculated on the clone sample were 2.9%, 8.2%, and 16.7% respectively.

The frequency of targeting to centromeric DNA was greatly increased in the hybrid cells, where the only residual human genetic material containing sequences homologous to pMR9A (used as a carrier for gene targeting) was the MC. Considering the presence of the human X chromosome translocated on a CHO chromosome in the hybrid and the partial homology among different chromosome specific  $\alpha$  satellite DNA sub-

families, chromosome X was included in our analyses. The calculated transfection efficiency was  $6 \times 10^{-5}$ . Twenty-four transfectants were isolated and analyzed for the presence of the MC, which was found in 8 of them. Of 116 scored mitoses, from 7 MC-positive clones, 79 showed the *neo* gene inserted into the MC centromeric region. The total number of available target sites was calculated on the basis of the equivalence of the total DNA content; it may therefore be presumed that human and CHO cells have the same total chromosome length. For the hybrid cells the median values of the  $Tf_{non\ cen}$ ,  $Tf_{cen\ 9\ MC}$ , and  $Tf_{cen\ MC}$  calculated on the clone sample were 0.4%, 25.0%, and 80.0% respectively.

The data obtained both for the HT1080 cell line and the hybrids was statistically analyzed using log-linear models (SPSS-PC+). This analysis demonstrated that clones were heterogeneous as far as the proportion of integrations at the different sites was concerned. The data for each clone was then analyzed independently. The increase in centromeric versus noncentromeric integration sites in 8 out of 10 HT1080 clones and in all (7/7) the hybrid clones turned out to be statistically highly significant ( $p < 0.00001$ ) either according to  $\chi^2$  or Fisher's exact test. Using the same approach, the increase in MC-specific centromeric versus nonspecific centromeric integration sites was demonstrated to be statistically highly significant ( $p < 0.00001$ ) in 3 out of 7 hybrid clones. In the remaining clones, the  $\chi^2$  or Fisher's exact test could not be found significant due to the limited size of the samples. However the majority of clones (9/10 for HT1080, 7/7 for hybrids) showed an increased frequency of integration events in centromeric versus noncentromeric regions on one side and in chromosome 9 (HT1080) or MC (hybrids) centromeric DNA versus other centromeric regions on the other. In all comparisons the low probability of the sign test  $\chi^2$  ( $p < 0.025$ ) confirmed that the tendency toward centromeric integration with preferential targeting to chromosome 9 or MC centromeric DNA cannot be considered to be due merely to random events.

One of the hybrid clones, Tr4, showing a single integration site on the MC (Fig. 2), was propagated in culture, both in the presence and in the absence of selective pressure, to test the mitotic stability of the MC after transfection. The data shown in Table 1 demonstrate an increased stability of the MC under selective pressure. However, in prolonging the culture, a sizeable fraction of cell population maintains the MC even without the selective conditions.

TABLE 1. MITOTIC STABILITY OF THE MC BEFORE AND AFTER TRANSFECTION

Culture days	Before transfection % MC <sup>+</sup> cells <sup>a</sup>	After transfection	
		+G418	-G418
5	65	80	55
20	50	92	79
50	20	93	76
100	5	93	74

<sup>a</sup>Calculated on samples of at least 50 metaphase spreads.

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Genomic DNA from the Tr4 clone and DNA from the YAC used for transfection were digested with *Pvu* I and probed either with pSV2neo or with the chromosome 9-specific  $\alpha$  satellite DNA (pMR9A) after electrophoresis and transfer to a nylon filter. The genomic DNA showed, when probed with pSV2neo, a hybridization profile identical to that of the control with two additional bands of lower molecular weight (Fig. 3, lane B). The additional bands of 1.25 and 2.1 kb, hybridized also with the pMR9A probe (Fig. 3, lane C). These results demonstrate that more than one copy of the intact YAC are inserted into the MC; the additional bands represent vector-host DNA junctions.

## DISCUSSION

The construction of YACs (Murray and Szostak, 1983; Gnirke *et al.*, 1993) and their exploitation as cloning vectors, has stimulated research on mammalian artificial chromosomes (MACs) (Brown, 1992; Willard and Davies, 1993; Sun *et al.*, 1994). Such kind of vectors may offer means for a new approach to the treatment of those genetic diseases in which the underlying defect is a deletion of a large portion of DNA. Examples include: DMD,  $\alpha$ -thalassaemia, and contiguous gene

syndromes (e.g., WAGR, Prader-Willy, Miller-Dieker, DiGeorge, Langer-Giedion syndromes). However, many problems still remain unsolved. Issues concerning the determination of the minimum chromosome size needed for a regular replication of DNA, its condensation and segregation, and the identification of DNA sequences necessary for full centromere function are the objects of ongoing studies.

To carry on investigations along these lines, we propose an approach based on the use of a model system represented by human supernumerary MC. Our results indicate that:

(i) A selectable marker gene can be targeted to the centromeric DNA of a human chromosome via co-transfection with chromosome-specific centromeric sequences, using monochromosomal somatic hybrids as recipient cells.

(ii) Once the gene has been inserted into the target centromeric sequences, the marker chromosome can be stably maintained in the absence of selective pressure, though at a reduced frequency.

(iii) This system might be considered as a potential tool for transferring foreign genes into mammalian cells, using autonomous elements at high capacity and containment.

To perform the co-transfection experiments, we produced a somatic cell hybrid containing the MC as the only centric human chromosome (Raimondi *et al.*, 1995). However, a fragment of the chromosome 6 and most of the X chromosome were also present and shown to be translocated on hamster chromosomes. These chromosome fragments apparently did not interfere with the efficiency of site-specific integration, owing to their lack of satellite sequences homologous to those used as a transfection carrier. In this respect, it is worth noting that the preliminary experiments that allowed us to identify the MC and to characterize its centromeric region played a key role in planning the best targeting procedure.

A notable feature of the results of co-transfection experiments is the high efficiency of gene targeting to the MC centromeric DNA. The data show a marked increase in the median value of the percentage of MC-specific centromeric integration sites in the hybrid clones compared with that observed in the HT1080 clones for chromosome 9, the values being 80% and 16.7%, respectively. The frequency of homologous recombination in mammalian cells depends on different factors, such as the extent of homology between exogenous DNA and target sequences, the length of the homologous stretches, as well as on the organization of the target DNA; the repetitive sequences are likely to be at an advantage (Capecchi, 1989; Hasty *et al.*, 1991). The presence of a high rate of random integration sites in the HT1080 cells was actually expected, due to the dilution of the target sequence, chromosome 9 centromeric DNA, among the homologous sequences localized on other chromosomes. In fact, it is known that in human cells all the centromeric sequences carry partially homologous  $\alpha$  satellite subfamilies (Willard, 1990; Tyler-Smith and Willard, 1993). In the hybrid clone, the only target site homologous to the carrier DNA used for transfection was the MC centromeric DNA.

In view of a possible use of the MC as a vector for gene therapy, an essential step was to check its stability in culture after transfection with and without selective pressure. The increased stability of the transformed MC in the absence of selective pressure could be related to the transfection procedure itself, based on the use of alphoid DNA as a carrier for gene

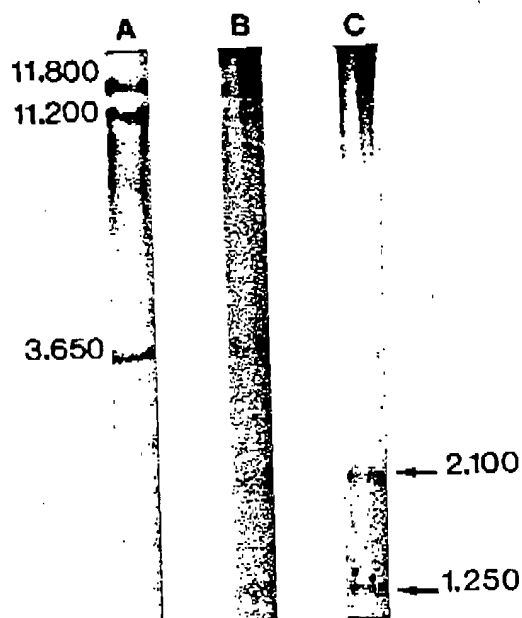


FIG. 3. Southern blot with the pSV2neo and with the pMR9A probes on *Pvu* I-digested DNA from the YAC used for transfection (lane A) and genomic DNA from the Tr 4 clone (lanes B and C). Three bands of 11.8 kb, 11.2 kb, and 3.65 kb can be seen in the lane corresponding to the original YAC hybridized with the pSV2neo probe (lane A). The same profile with two additional bands of 2.1 and 1.25 kb can be observed in the lane corresponding to Tr 4 genomic DNA (lane B) hybridized with the same probe. These additional bands also hybridize with the pMR9A probe (lane C); this indicates that they correspond to YAC-host DNA junctions.

targeting. The insertion of these sequences probably led to an increased size of the centromeric satellite DNA. Considering the high frequency of chromosomal rearrangements generally observed in human/hamster cell hybrids, the hypothesis of a translocation of hamster centromeric sequences on the MC can be considered. This would again result in the increased stability of the MC. However, the proof that the integrity of the transferred sequences is maintained after transfection makes this hypothesis less plausible.

Our results suggest that gene targeting to the MC centromeric DNA does not alter the essential components that allow the MC to replicate and segregate normally during cell propagation *in vitro*. On the contrary, its mitotic stability appears to be increased. This represents an instrumental condition for a successful transfer of the MC into different kinds of recipient mammalian cells.

Few other reports have been published concerning MC stably maintained in mammalian cells. Law *et al.* (1982) and Carine *et al.* (1986) described MC containing the centromeric region of chromosomes 12 and 1, respectively. In both cases, these MC were obtained from X-irradiated monochromosomal cell hybrids. Hamkalo *et al.* (1985) observed a MC containing mouse satellite DNA in NIH-3T3 cells, presumably originated from a normal mouse chromosome by a massive deletion and telomere addition. More recently, Brown *et al.* (1994) were able to obtain a MC containing the short arm and part of the centromeric sequences of the human Y chromosome by means of telomere-directed chromosome fragmentation. In all of these cases, the MC were derived by size reduction of normal chromosomes and were likely to carry a number of single-copy genes that could cause gene dosage imbalance. The MC described in the present paper is recognized as an accessory element in a human karyotype and does not seem to be correlated with the pathological symptoms observed in the donor patient, traceable to the complete trisomy 9; moreover, the MC appears to be devoid of any intrachromosomal effect. This suggests that the extra copies of genes present in the MC may not impair normal cell functions.

The MC is currently being size-reduced by means of radiation fragmentation (Lawrence *et al.*, 1991) and telomere-directed chromosome fragmentation (Farr *et al.*, 1991). Size reduction is a key step in manipulating the MC into yeast cells and in transferring it into mammalian somatic cells. The strategy we are applying would enable us to overcome some of the difficulties that hinder the construction of MACs as potential vectors for gene therapy. An important point will be the determination of the *in vitro* and *in vivo* adaptive value of a manipulated MC. It will also be crucial to follow the *in vitro* and *in vivo* expression of transgenes, targeted into "MAC."

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